DENTAL CARIES IN THE COTTON RAT

XIII. THE EFFECT OF WHOLE GRAIN AND PROCESSED CEREALS ON DENTAL CARIES PRODUCTION[1]

MARGUERITE A. CONSTANT, PAUL H. PHILLIPS AND C. A. ELVEHJEM

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

THREE FIGURES

(Received for publication August 24, 1951)

INTRODUCTION

Cotton rats have been found to be highly susceptible to the cariogenic action of a soft and finely ground natural diet consisting of oatmeal, dried whole milk and sugar (Constant et al., '51). Greater tooth destruction resulted from feeding lower levels of the dried whole milk, either by increasing the cereal level or by substituting casein and dextrin for the milk portion. It seemed likely that the cereal and mineral contents of our natural diet were the factors primarily concerned in its extensive cariogenicity. The consumption of processed cereals has been considered by several investigators to be an important factor in the increased caries incidence of many peoples who previously had been relatively caries-free (Osborn and Noriskin, '37; Stephan, '48). We wish to report further studies on the caries-producing activity of processed or whole cereals when fed to cotton rats.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Nutrition Foundation, Inc., New York, N. Y., and the National Dairy Council, Chicago, Ill.

We are indebted to Merck and Co., Rahway, New Jersey, for the crystalline vitamins and to Abbott Laboratories, North Chicago, Illinois, for the halibut liver oil.

Copyright, 1952
The Wistar Institute of Anatomy and Biology
All rights reserved

EXPERIMENTAL AND RESULTS

Weanling cotton rats (19 to 21 days of age) from our stock colony were used in these experiments. Stock animals and young were maintained on a non-cariogenic stock diet. Weanlings were distributed by litter-mate pairs among the control and experimental groups because of the observations made by Schweigert et al. ('45) that cotton rats from different parental stock varied in their susceptibility to cariogenic diets. All animals were fed and watered ad libitum and were given two drops of haliver oil each week.

In the first study the cariogenicity of the oatmeal diet was compared with that of a partially synthetic, high sucrose diet. The partially synthetic diet contained 67% sucrose, 24% casein, 4% salts IV (Phillips and Hart, '35, patterned after the salts of milk), 5% corn oil, adequate levels of B vitamins (Shaw et al., '44) and 4% 1:20 liver extract. The cereal diet consisted of 50% oatmeal, 32% dried whole milk and 18% sucrose. The total sugar content of this diet was 30% (18% sucrose and 12% lactose). The animals were kept on experiment 14 weeks.

The dental caries scores of these animals are given in table 1. Both diets were equally acceptable to the animals, which showed normal and comparable weight gains. Contrary to expectations, the caries scores revealed that the 67% sucrose diet was less cariogenic than the cereal-milk diet. When the 67% sucrose diet was fed, there were 38% fewer carious fissures and a 50% reduction in the severity of the lesions. These results suggested that the cereal part of the ration contributed to its cariogenicity.

A second study was then conducted to determine whether the cariogenicity of the cereal used was characteristic of cereals in general. Various whole grain or processed cereals were fed at a level of 50% in the above cereal diet. Litter-mate pairs were fed whole corn or corn flakes, whole wheat or wheat flakes, and whole oats, with control animals receiving oatmeal (4 to 5 pairs in each group). In order to maintain

TABLE 1

The effect of whole grain or processed cereals on caries production

RATION FED	NO. OF LITTER-MATE PAIRS	CARIOUS LESIONS [1]						CARIOUS LESIONS (Corrected for fractures)	
		Average incidence ± S.E.M.		Calc'd. "t"	Average extent ± S.E.M.		Calc'd. "t"	Average incidence	Average extent
Study 1									
Oatmeal basal	8	18.5 ± 3.3	>	4.9	54 ± 10	>	6.2	14	36
67% sucrose diet		11.0 ± 2.6			24 ± 8			8	12
Study 2									
Oatmeal basal		18.5 ± 1.9	>	5.2	55 ± 7	>	3.5	13	35
Processed cereals	14	18.6 ± 2.9	×		64 ± 10	×		9	25
Whole grains		7.1 ± 2.2	<	3.7	25 ± 9	<	4.2	3	6
Oatmeal basal	10	18.6 ± 1.6	>	0.1	54 ± 7	>	1.0	14	34
Processed cereals		18.8 ± 3.5			67 ± 14			8	20
Study 3									
Dextrin basal	9	25 ± 1.9	≥	3.3	76 ± 7	≥	2.8	18	45
with floured potato	9	20 ± 1.4	≥×	2.0	51 ± 7	≥×	2.3	16	38
with potato and acid	6	23 ± 1.5	≥×	0.7	69 ± 3	≥×	1.7	14	30
Dextrin basal	9	25 ± 1.9	≥	3.6	76 ± 7	≥	4.4	18	45
with oatmeal	9	14 ± 2.1	≥×	2.9	27 ± 5	≥×	4.8	13	23
with oatmeal and base	6	16 ± 3.4	≥×	0.3	34 ± 9	≥×	0.5	14	26
Dextrin basal	5	20 ± 1.9	≥	2.1	60 ± 9	≥	1.5	14	32
with wheat flour	5	16 ± 2.0	×		42 ± 7	×		13	29
with floured rice	4	21 ± 2.5	≥	0.6	57 ± 9	≥	0.2	15	30
with rice and base	4	21 ± 3.1	≥	0.1	52 ± 14	≥	0.6	17	34

[1] Incidence equals average number of carious fissures per capita. (The cotton rat has a total of 40 fissures per head, 10 in each quadrant.) Extent is an expression of the average total caries involvement per capita. Individual lesions are evaluated 1^+ to 5^+ with increasing severity of lesions; 1^+ and 2^+ = lesions involving primarily the enamel; 3^+ = enamel and a considerable amount of dentine; 4^+ and 5^+ = lesions resulting in fractures.

diets of similar consistency and particle size, the whole oat or wheat grains and oatmeal were ground in a Wiley mill until they passed through a fine screen (approximately 60 mesh). The more brittle and fracturing cereals, whole corn and corn or wheat flakes, were reduced to a flour in a ball mill. The experimental period was 14 weeks.

With the exception of whole oats or corn flakes, which resulted in a decreased growth rate, the feeding of the various cereals in the cereal-milk diet resulted in good growth of the animals. That the difference in growth was not part of the caries picture was shown when the teeth were evaluated for caries. The teeth of the animals fed corn flakes were extensively carious, while those of the animals fed whole oats had little decay. The caries scores of the animals fed the whole grain or processed cereals are summarized in table 1. These data revealed that whole grains were less cariogenic than the processed cereals. The average number and extent of the carious fissures were 50% less when the diet contained whole grains than when it contained processed cereal products. The variation in caries scores when the diets contained whole grains and the increased number of fractured cusps when the diet contained corn or wheat flakes suggested that some of the caries found accompanying the fractured cusps may have been due directly to the fracture, which was an uncontrollable physical variant. The data were recalculated to evaluate the caries involvement of intact teeth by eliminating the caries in fractured cusps from the total score. These corrected caries scores, given in table 1, did not alter the caries evaluation. The diets which contained processed cereals still resulted in considerably more carious fissures, excluding those in fractured cusps. Thus the extensive cariogenicity of oatmeal was shown to be a general characteristic of processed cereals. It should be pointed out that these experiments indicate that the cariogenicity of a given diet is not contingent solely upon the type and amount of carbohydrate used.

Larsen ('49) observed that Hawaiian natives eating taro (alkaline ash) generally had considerably less tooth decay

than those consuming rice (acid ash). Therefore a further study was made of the cereal portion of the ration by comparing the cariogenicity of acid residue cereals with that of alkaline residue foods when each was incorporated in a partially synthetic diet as the major source of carbohydrate. The basal diet for these experiments was formulated to approximate the composition of the cereal-milk diet used in the previous experiments. It consisted of 17% casein, 5% cellu flour, 53% floured dextrin, 18% sucrose, 2% salts IV, 5% corn oil, and adequate levels of B vitamins. The various cereals used were substituted for the floured dextrin of the basal diet. In order to maintain approximately equivalent levels of protein and carbohydrate, the casein level was decreased to 12% and the amount of cellu flour raised correspondingly to 10% in the experimental diets.

Potatoes were used as representative of an alkaline-ash food. These were cooked, peeled, dried, and then reduced to a flour in a ball mill. Wheat flour, rice flour, and finely ground oatmeal were used as representative acid-ash foods. The ash character of some of these foods was altered by the addition of acid or base. An aqueous solution of 1 N HCl or 1 N NaOH was dispersed by pipette through the cereal in such quantity as to provide an excess of 0.1 equivalent of acid or base per 100 gm of cereal. The treated cereals were then dried and reground before being mixed in the diet. These diets were fed for from 10 to 12 weeks.

The caries scores given in table 1 showed that the cariogenic activities of potatoes, rice, wheat and dextrin were similar. Under the conditions of these experiments, oatmeal produced less caries than the other foods. It is of interest to point out and emphasize that oatmeal was the only food which was not reduced to a flour, and therefore was of coarser particle size. The destructive effect of the basal diet, whose largest particles were provided by the casein, is shown in figure 1. Altering the ash character of these foods by the addition of acid or base did not alter their cariogenicity. Hence, the acid or alkaline ash residue effect of the foods studied

had no measurable influence upon the cariogenicity of these foods.

The lack of plaque material on the teeth of the above animals (fig. 1) showed that the heavy yellow-brown material and the deep yellow-brown pigmentation of the lesions associated with the feeding of the 67% sucrose diet (fig. 2) were primarily due to inclusion of liver extract in the latter diet. The marked degree of softening of the wide enamel portion of the tooth, the paleness of the lesions and the "bleached" appearance of the teeth (fig. 3) have been found to date to be

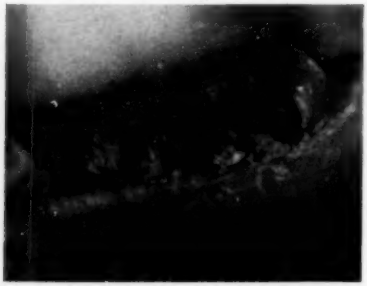

Fig. 1 Mandibular teeth of a cotton rat fed the basal ration containing dextrin in the form of a fine flour, without liver extract.

associated with the feeding of diets with a high oatmeal and low mineral content.

DISCUSSION

It is evident from the experimental procedure used that the cotton rats in these studies constantly consumed the same diet as their only source of nutrients for the entire experimental period. In this respect the results obtained from these studies cannot be applied directly to caries production in humans, for one would not expect humans to consume these proportions of foodstuffs each meal of the day, or for several days. However, the basic findings of these studies — that is, the greater cariogenicity of processed cereals and the unpre-

dictability of the cariogenicity of diets on the basis of their carbohydrate content — apply rather universally to caries in the human.

In a review of various saccharides and dental caries studies, Stephan ('48) discussed several *in vitro* experiments which showed that not all *Lactobacillus* organisms fermented lactose under the given conditions, and that oral microorganisms produced pH changes more slowly with lactose than other disaccharides. Polysaccharides were much more inert. Ac-

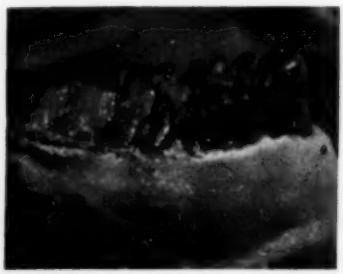

Figure 2

Figure 3

Fig. 2 Mandibular teeth of a cotton rat fed a synthetic diet containing 4% liver extract powder.

Fig. 3 Mandibular teeth of a cotton rat fed a low mineral oatmeal diet without liver extract. Note softness of outer cusp, which permitted easy penetration by a no. 42 fine wire dental probe.

cording to these findings, the natural diet containing processed cereals used in these experiments should have been less cariogenic than the synthetic diet. Both diets contained 67% carbohydrate but in the synthetic diet all the carbohydrate was present as sucrose, whereas in the natural diet only 30% was sugar (18% sucrose, 12% lactose). However, in the course of investigating the role of nutrition in caries production in cotton rats in this laboratory, Zepplin et al. ('50) observed that natural diets containing 17% sucrose were more cariogenic than a synthetic diet containing 67% sucrose. The nat-

ural diets used by Zepplin et al. resembled the average human dietary as closely as possible within the limitations imposed by the use of experimental animals. The results of the present study confirm those of Zepplin et al. and further support the observation made previously (Constant et al., '51) that under certain conditions complex carbohydrates may be as cariogenic as simple sugars.

Work in this laboratory has shown that the physical state of the diet (Anderson et al., '48; Smith, '48; Constant et al., '51), and the ratio of cariogenic to anti-cariogenic nutrients (Schweigert et al., '46; Constant, '51), as well as the total amount of carbohydrate or fermentable sugar, may alter the extent of caries production. The protein, carbohydrate, and fat levels of the natural and the 67% sucrose diets employed were similar. Smith ('48) found that dextrin in the form of a fine flour resulted in approximately twice as many carious fissures as less finely ground dextrin. In addition to the effect of different particle sizes and impaction, Hoppert et al. ('32) noted the influence of another type of retention due to plasticity and adhesiveness. Data obtained in this laboratory (Constant, '51) have shown that higher levels of calcium or the feeding of highly basic salts considerably reduced caries production by high sucrose or high cereal diets. It is of interest to point out that the 67% sucrose diet contained twice as much minerals as the natural diet. Although the influence of the differences in the physical characters of the natural and the synthetic diets cannot be properly evaluated at this time, it is suggested that the primary cause of the greater cariogenicity of the natural diet is the decreased buffering and neutralizing capacity of this diet. This is either due to its lower mineral content or the unavailability of the minerals present.

With respect to the greater cariogenicity of processed cereals in comparison to whole grains, Osborn and co-workers (Osborn et al., '37; Osborn and Noriskin, '37) concluded as a result of *in vivo* and *in vitro* experiments that this greater

cariogenicity was due to the loss of minerals, particularly calcium and phosphorus. The mineral content of the various cereal diets used by us was very similar, due to the presence of 32% milk. This level of milk (providing minerals approximately equivalent to 2% salts IV) was sufficient to result in a normal ash content of the femurs (63 to 67%). The diets were essentially identical except for the type of cereal fed. Although the local action of minerals intimately associated with the carbohydrate portion of the diet cannot be ignored, the results of the present studies suggest that during the refining process the complex carbohydrates are dextrinized to such an extent that processed cereals are more readily available to and utilized by caries-producing organisms. We also cannot exclude at this time the possibility that refining processes may be exerting an influence by other means, such as the destruction of natural antibiotics or enzyme factors. Additional study is being made of natural and processed foods in order to elucidate further their influence on the dental caries process.

SUMMARY

The effect of whole grain versus processed cereals and alkaline-ash versus acid-ash foods on the experimental production of dental caries in the cotton rat has been studied.

When fed under similar conditions, the whole grain cereals, corn, wheat and oats, were found to be less cariogenic in each case than their processed products — corn flakes, wheat flakes and oatmeal, respectively.

Natural diets which contained processed cereals and only 30% sugar were found to be more cariogenic than a synthetic diet containing 67% sucrose. The reasons for this rather unexpected finding have been discussed.

A significant difference was not found between the cariogenicity of alkaline-ash and that of acid-ash foods under the experimental conditions used.

Changes in the appearance of the teeth and the carious lesions, due to dietary ingredients, have been noted.

LITERATURE CITED

ANDERSON, E. P., J. K. SMITH, C. A. ELVEHJEM AND P. H. PHILLIPS 1948 Dental caries in the cotton rat. X. The effect of fluidity of the ration. J. Nutrition, *35:* 371–377.

CONSTANT, M. A. 1951 Studies of factors influencing the production of dental caries and tissue calcification in the cotton rat. Ph.D. thesis, University of Wisconsin.

CONSTANT, M. A., P. H. PHILLIPS AND C. A. ELVEHJEM 1951 Dental caries in the cotton rat. XII. Natural versus refined sugars. J. Nutrition, *43:* 551–564.

HOPPERT, C. A., P. A. WEBER AND T. L. CANIFF 1932 The production of dental caries in rats fed an adequate diet. J. Dent. Res., *12:* 161–173.

LARSEN, N. P. 1949 Tooth decay in relation to diet and general health. J. Am. Med. Assn., *137:* 832–838.

OSBORN, T. W. B., AND J. N. NORISKIN 1937 The relation between diet and caries in the South African Bantu. J. Dent. Res., *16:* 431–442.

OSBORN, T. W. B., J. N. NORISKIN AND J. STAZ 1937 Inhibitin *in vitro* of decalcification in teeth. Ibid., *16:* 545–550.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657–663.

SCHWEIGERT, B. S., J. H. SHAW, C. A. ELVEHJEM AND P. H. PHILLIPS 1945 Dental caries in the cotton rat. V. Influence of strain variation on the caries susceptibility. Proc. Soc. Exp. Biol. Med., *59:* 44–47.

SCHWEIGERT, B. S., J. H. SHAW, M. ZEPPLIN AND C. A. ELVEHJEM 1946 Dental caries in the cotton rat. VI. The effect of the amount of protein, fat, and carbohydrate in the diet on the incidence and extent of carious lesions. J. Nutrition, *31:* 439–447.

SHAW, J. H., B. S. SCHWEIGERT, J. M. MCINTIRE, C. A. ELVEHJEM AND P. H. PHILLIPS 1944 Dental caries in the cotton rat. I. Methods of study and preliminary nutritional requirements. J. Nutrition, *28:* 333–345.

SMITH, J. K. 1948 Physical state of the ration influencing dental caries in the cotton rat. M.S. Thesis, University of Wisconsin.

STEPHAN, R. M. 1948 Relative importance of polysaccharides, disaccharides and monosaccharides in the production of caries. J. Am. Dent. Assn., *37:* 530–536.

ZEPPLIN, M., J. K. SMITH, H. T. PARSONS, P. H. PHILLIPS AND C. A. ELVEHJEM 1950 Dental caries in the cotton rat. XI. The effect of feeding a natural diet comparable to a human diet. J. Nutrition, *40:* 203–211.

THE EFFECT OF VITAMIN B_{12} DEFICIENCY UPON THE SURVIVAL OF YOUNG BORN TO RATS FED PURIFIED CASEIN RATIONS

LESLIE P. DRYDEN, ARTHUR M. HARTMAN AND CHARLES A. CARY[1]
Bureau of Dairy Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

(Received for publication October 1, 1951)

The performance of rats fed certain purified casein rations has been previously described in regard to fertility of the female and birth weight of the young (Dryden, Hartman and Cary, '51). It has likewise been reported briefly that young born to mothers fed these rations failed in many instances to survive more than a few days after parturition and that this condition could be corrected by feeding to the mothers a separate supplement of 15 U./ml antipernicious anemia liver extract or crystalline vitamin B_{12} (Hartman et al., '46; Cary and Hartman, '48; Dryden, Hartman and Cary, '49; Hartman, Dryden and Cary, '49). Casein rations equally or nearly equally deficient in vitamin B_{12} as determined by rat growth assay have been found to vary somewhat in their tendency to produce early deaths of newborn young; survival of young from birth to weaning on these various rations is considered in the present paper.

EXPERIMENTAL PROCEDURE

The rats used in these experiments (other than the F_1, F_2 and F_3 generations of experiment 4) were stock colony mothers that had given birth to one or more litters prior to being placed on the experimental rations. Each mother, together

[1] Formerly Head, Division of Nutrition and Physiology; retired February, 1950.

with her litter, was placed at parturition or a day or so thereafter upon the experimental diet and, after the young were weaned, was continued on the experimental ration for a number of successive matings.

In the first three experiments and in the 5th experiment, when the litters contained more than 6 young at the end of one week after birth, they were reduced to 6 at that time; in the 4th experiment, they were reduced to 6 at birth, a procedure that was likewise followed in the stock colony. The young retained were weaned at 25 days of age.

A number of B_{12}-deficient rations [2] were used in these experiments. All of them were modifications of B_{12}-deficient ration 45, which consisted of 55.5% dextrin, 20.0% casein (10 × hot alcohol-extracted),[3] 10.0% dried brewers' yeast, 4.5% salts, 9.85% cottonseed oil and 0.15% fish liver oil. The essential modifications are indicated in the tables in connection with the ration numbers. All rations and distilled water were supplied ad libitum.

Other experimental details and the composition of all the rations have been previously reported (Dryden, Hartman and Cary, '51).

In the first and second experiments, the performance of mothers placed on the vitamin B_{12}-deficient rations was followed for a few successive matings. Then some of these mothers who failed to raise any of their young, or at least (in experiment 2) any of the young from their last litter, were given an oral supplement in experiment 1 of 15 U./ml APA liver extract (0.10 ml/day) and in experiment 2 of crystalline vitamin B_{12} [4] (1 μg/day).

In the third experiment, primiparous stock colony mothers were put at parturition either on a vitamin B_{12}-deficient ration or on the same ration plus a supplement of 1 μg/day

[2] The crystalline folic acid used in some of these rations was kindly supplied by Dr. E. L. R. Stokstad and Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

[3] For preparation of casein, see Hartman, Dryden and Cary ('51).

[4] The crystalline vitamin B_{12} was kindly supplied by Dr. D. F. Green and Dr. M. A. Schooley, Merck and Co., Inc., Rahway, N. J.

of crystalline B_{12}. The 5th experiment was carried out similarly except that the mothers were not always primiparous and that the dose of crystalline vitamin B_{12} administered was 2 μg/day.

In the 4th experiment, 6 stock colony mothers were placed at parturition on a B_{12}-deficient ration. Some of the young (F_1 generation) that resulted from matings subsequent to the transfer to the B_{12}-deficient ration were maintained on a similar ration after weaning, while others were put at 28 days of age on the latter ration plus a supplement of 15 U./ml liver extract. These two groups were carried through the F_2 and F_3 generations, every mother receiving the same diet as its parents. In the F_1, F_2 and F_3 generations, B_{12}-deficient males were mated to B_{12}-deficient females and B_{12}-supplemented males to B_{12}-supplemented females.

RESULTS AND DISCUSSION

The survival record of the young in the first three experiments is shown in table 1. When the mothers were maintained on the stock ration until parturition, the survival of the young during the ensuing lactation was at least as good on the experimental rations as on the stock diet, regardless of the ration or experiment involved; furthermore, vitamin B_{12}-deficient mothers raised as large a proportion of their young to weaning as did vitamin B_{12}-supplemented mothers.

When the mothers were continued on the experimental rations through additional matings, however, quite different results were obtained. No young at all survived on some of the yeast-free B_{12}-deficient rations in two experiments, and indeed on none of such rations in any of the three experiments was there better than 39% survival. Where survivals did occur, they were confined to a few litters and a few mothers in each group. Of the 48 mothers on the yeast-free B_{12}-deficient rations, only 10 carried any young to weaning. Of these 10 mothers, one had only one litter born on the deficient ration, 4 others had surviving young only from the first litter, and another failed to have surviving young after

TABLE 1

Effect of vitamin B_{12} deficiency upon survival of young

RATION OF MOTHER [1]		EXPT. NUMBER	SUPPLEMENT	NUMBER OF MOTHERS [2]	NUMBER OF LITTERS [3]	YOUNG		
During gestation	During lactation					Total number nursed by mothers [4]	Per cent dying during first week	Per cent weaned
Stock	Stock	..	None	..	..	23,648	..	88
Stock	Exptl.	1,2,3	None	84–84	69–15–0	504	2	96
Stock	Exptl.	3	B_{12}	26–26	23–3–0	156	2	98
195 (Yeast-free; added B vits., E, K, C, methionine)	192	1	None	9–0	0–0–17	110	100	0
		2	None	10–3	1–4–9	60	63	23
		3	None	5–0	0–0–16	78	100	0
		1	Liv. ext.	4–4	4–4–0	51	14	84
		2	B_{12}	5–4	6–1–3	32	12	84
		3	B_{12}	7–5	9–3–3	60	12	80
196 (Yeast-free; 2 × 192–195 level vits. and methionine)	193	1	None	8–3	3–2–9	75	56	39
		2	None	8–2	3–1–8	47	74	26
		1	Liv. ext.	4–4	3–4–1	43	13	84
		2	B_{12}	5–4	6–1–1	31	3	90
301 (Yeast-free; added B vits., E, K, C; 15% lactose)	300	3	None	5–1	0–1–9	53	85	11
		3	B_{12}	3–3	6–1–0	41	5	95
303 (Yeast-free; added B vits., E, K, C; 1% succinylsulfathiazole)	302	3	None	3–1	0–1–4	29	86	14
		3	B_{12}	4–4	5–2–0	41	5	93
197 (Yeast; added E, K, C, methionine)	194	1	None	9–6	4–4–16	145	78	21
		2	None	9–9	10–5–4	89	16	72
		3	None	8–7	7–7–6	99	30	63
		1	Liv. ext.	2–2	2–1–0	14	7	93
		2	B_{12}	1–1	0–1–0	5	0	60
		3	B_{12}	9–9	14–4–4	88	10	86

[1] The experimental rations fed during gestation and those fed during lactation were similar except that the former contained 25% and the latter 30% protein.

[2] The first figure indicates the total number of mothers that had living young found at birth; the second figure, the number that had some young surviving to weaning.

[3] The first figure indicates the number of litters in which all young survived to weaning; the second figure, the number in which some young survived to weaning; the third figure, the number in which no young survived to weaning.

[4] These figures do not include those rats discarded at the end of the first week in reducing the litters to 6 rats each, but the percentage of young dying in the first week is based on the total number of young found living.

the second litter. The other 4 mothers, however, showed no tendency toward a decreased percentage of surviving young with later litters. Thus, while the occurrence of mothers who had surviving young might be explained partly on the basis of greater storage of vitamin B_{12} from the stock colony ration, it would appear that some animals must either have synthesized B_{12}-active material in the intestinal tract or else have had some other natural resistance to the deficiency (see Hartman et al., '51, for evidence in regard to synthesis of B_{12}-active material in growing rats on some of these rations).

With the 5th experiment somewhat different results were obtained on the yeast-free rations. The performance of these animals is shown in table 2. Survival in the first litters born while the mothers were on ration 195 was not impaired, but from the second litters no young at all survived. With ration 443, which differed only slightly from rations 192–195, survival was somewhat poorer in the first litters and better in the second litters than had been observed on rations 192–195. Such results in contrast to those of the first three experiments, indicate a considerably increased carry-over effect from the stock ration or increased intestinal synthesis of B_{12}-active material, or both.

It would seem that the somewhat improved survival of the second litters born on ration 443 as compared to those born on rations 192–195 was probably not due to the small amount of lactose included in the ration (compare rations 300–301 with rations 192–195 in table 1). It would also appear (table 1) that the inclusion of succinylsulfathiazole (1%) in rations 302–303 produced results that were no different from those obtained on rations that did not contain this bacteriostatic agent. This latter observation is somewhat in contrast to that of Schultze ('50a) who, with a ration containing a commercial soybean protein, found that the early deaths of newborn young which had previously been observed were largely eliminated when a sulfa drug (2% phthalylsulfathiazole) was incorporated in the ration.

TABLE 2

Survival of young and food consumption during gestation of mothers on B_{12}-supplemented and unsupplemented B_{12}-deficient rations
(Experiment 5)

RATION OF MOTHER [1]		NO. OF LITTER BORN ON EXPTL. RATION	SUPPLEMENT	NUMBER OF MOTHERS [2]	NUMBER OF LITTERS [3]	YOUNG			FOOD CONSUMPTION DURING GESTATION
During gestation	During lactation					Total number nursed by mothers [4]	Per cent dying during first week	Per cent weaned	
									gm/day
Stock	Exptl.	..	None	29–29	28–1–0	176	0	99	...
Stock	Exptl.	..	B_{12}	15–14	12–2–1	89	0	88	...
195	192	1	None	5–5	4–1–0	29	3	97	14.6 ± 0.65 [5]
195	192	2	None	3–0	0–0–3	19	100	0	13.9 ± 0.56
195	192	1	B_{12}	8–7	7–0–1	44	5	86	14.0 ± 0.56
195	192	2	B_{12}	5–5	3–2–0	23	22	74	14.8 ± 1.14
443	443	1	None	7–6	4–2–1	41	27	71	13.7 ± 0.46
443	443	2	None	7–5	2–3–2	43	51	46	12.5 ± 0.36
443	443	1	B_{12}	6–5	4–1–1	33	0	79	13.1 ± 0.37
443	443	2	B_{12}	4–3	3–0–1	23	15	78	13.6 ± 0.59
445	445	1	None	4–4	1–3–0	30	9	80	12.5 ± 0.38
445	445	2	None	5–3	1–2–2	34	54	35	12.5 ± 0.44
195 or 443	192 or 443	1	40 × Mn [6]	12–6	2–4–6	51	59	29	14.0 ± 0.39
195 or 443	192 or 443	2	40 × Mn	8–5	0–5–3	47	62	34	14.3 ± 0.57

[1] Rations 443 and 445 were similar to ration 192 except that 443 contained 1.5% lactose and 445 contained 15% lactose and 20% yeast.

[2] The first figure indicates the total number of mothers that had living young found at birth; the second figure, the number that had some young surviving to weaning.

[3] The first figure indicates the number of litters in which all young survived to weaning; the second figure, the number in which some young survived to weaning; the third figure, the number in which no young survived to weaning.

[4] These figures do not include those rats discarded at the end of the first week in reducing the litters to 6 rats each, but the percentage of young dying in the first week is based on the total number of young found living.

[5] Standard error of the mean.

[6] Enough $MnSO_4 \cdot 4H_2O$ added to the ration to bring the total level of added manganese to 40 times that in the other rations.

Increasing the levels of B vitamins (other than B_{12}) in the ration did not appear to have any effect in increasing the survival of the young. While rations 193–196, which contained double the levels of vitamins contained in rations 192–195, appeared to give somewhat better results in the first experiment, no material difference was observed in the second experiment (table 1).

Since the early deaths of the young resembled to some extent the phenomena observed among rats on diets containing very low levels of manganese (Shils and McCollum, '43, and others), groups on B_{12}-deficient rations containing a greatly increased level of added manganese were included in experiment 5 (table 2). It can be seen, however, that the added manganese did not improve survival. In fact, it appeared to bring on the deficiency more rapidly, as evidenced by the poor survival of young in the first litters born on these rations as compared to survival on the same rations without the additional manganese.

The feeding of crystalline vitamin B_{12} as a supplement to yeast-free B_{12}-deficient rations (tables 1 and 2) prevented these early deaths in most cases. There were still, however, a few rats (about 15%) who were unable to carry their young to weaning even though receiving a large quantity of vitamin B_{12}.

With mothers on B_{12}-deficient yeast-containing rations, survival of the young to weaning was in some experiments (1 and 5) very little different from that observed on the yeast-free rations; in others (2 and 3) survival was much better on the yeast rations. In the latter two experiments almost all the mothers had surviving young and, while the per cent survival was in general somewhat below normal, it was not lowered to as great an extent as with the yeast-free rations. Such a difference in results between experiments may possibly be explained by differences in the lots of yeast used in the various experiments. In practically all cases, vitamin B_{12}-active material, where tried, either restored to normal or prevented any depression below normal of the

survival rate. It seems clear, then, that when the rations were B_{12}-deficient, the yeast-containing rations often produced much better survival records than did the yeast-free ones. When these rations were supplemented with vitamin B_{12}, the differences were much less striking, although there was still apparently a tendency for a larger percentage of mothers on the yeast-containing rations to have survivors than those on the diets devoid of yeast.

TABLE 3

Effect of stock young being nursed by deficient mothers and deficient young by stock mothers and of injecting deficient young at birth with vitamin B_{12}-active material

NUMBER OF MOTHERS	NUMBER OF LITTERS	YOUNG		
		Number nursed by mothers	Per cent dying during the first week	Per cent weaned
Stock young nursed by deficient mothers:				
17	24	130	14	85
Young born to deficient mothers but nursed by stock mothers:				
5	5	15	60	20
Deficient young injected at birth with crystalline B_{12} or liver extract:				
Group A (litters where some of non-injected deficient young survived):				
	Non-injected young:	9	67	33
2	2			
	Injected young:	6	0	100
Group B (litters where none of the non-injected deficient young survived):				
	Non-injected young:	5	100	0
3	3			
	Injected young:	6	100	0

Almost all the deaths of young born to mothers on the B_{12}-deficient rations occurred during the first three or 4 days after birth. Death was evidently not due to a failure of the mothers to lactate, for in most cases considerable amounts of milk were observed in the stomachs of the dead young. In addition, it is apparent in table 3 not only that stock young transferred for nursing to deficient mothers (who, except in two instances, had lost their own young) were

carried to weaning in about the same proportion as stock young nursed by stock mothers (table 1), but also that deficient young nursed by stock mothers survived no better than when nursed by their own mothers.

A few young from deficient mothers were injected at birth with small amounts of crystalline vitamin B_{12} or APA liver extract. On the basis of the data in table 3 it would seem probable that whether or not such injections can prevent death depends upon the severity of the deficiency.

In table 2 the average daily food consumption is given for the gestation periods prior to first and second parturitions on each of the rations involved. No significant differences between B_{12}-deficient and B_{12}-supplemented rations were observed. Moreover, a comparison of the average food consumption during the second gestation of all unsupplemented mothers none of whose young survived and all B_{12}-supplemented mothers all of whose young survived to weaning showed no significant difference ($t = 0.7$). Thus it seems evident that the failure of the young to survive on the B_{12}-deficient rations cannot be explained on the basis of a decreased intake by the mothers of some other ingredient of the ration.

It was of interest to see if any further decline in survival rate would occur in later generations of rats maintained on these B_{12}-deficient rations. Such data for yeast-containing rations are provided by the 4th experiment (table 4). It can be seen that the performance in the parent generation was much the same as that found in the other experiments, where good survival records occurred on yeast-containing rations. The over-all survival was 77%, somewhat higher than that obtained on the yeast rations in any of the first three experiments.

Of the original 6 mothers, one had no living young, another had no surviving young and a third had no surviving descendants beyond the F_1 generation. Descendants from only three mothers survived through every generation. Of these three lines, one (534) showed practically no impairment, sur-

TABLE 4

Survival of young as affected by maintaining rats on yeast-containing vitamin B_{12}-deficient casein rations through several generations

(Experiment 4)

MATERNAL LINE	GENERATION	RATION [1]	SUPPLEMENT	NUMBER OF MOTHERS [2]	NUMBER OF LITTERS [3]	YOUNG		
						Total number nursed by mothers [4]	Per cent dying during first week	Per cent weaned
454 [5]	Parent	Stock	None	1–1	3–0–0	18	..	100
454	Parent	47	None	0–0	.....	0	..	..
511	Parent	Stock	None	1–1	1–1–0	9	..	89
511	Parent	47	None	1–0	0–0–2	10	90	0
509	Parent	Stock	None	1–1	1–1–0	12	..	83
509	Parent	47	None	1–1	0–1–0	6	0	83
509	F_1	45–47	None	1–0	0–0–1	2	100	0
509	F_1	45–47	Liv. ext.	0– [6]	.....	..	..	..
467	Parent	Stock	None	1–1	1–1–0	18	..	94
467	Parent	47	None	1–1	2–0–0	10	0	100
467	F_1	45–47	None	2–1	1–0–9	50	90	10
467	F_2	45–47	None	3–3	7–2–0	48	6	92
467	F_3	45–47	None	8–8	20–5–2	137	12	86
467	F_1	45–47	Liv. ext.	2–2	6–1–1	42	7	93
467	F_2	45–47	Liv. ext.	3–3	8–4–0	68	4	93
467	F_3	45–47	Liv. ext.	2–2	6–1–0	42	2	98
588	Parent	Stock	None	1–1	1–0–0	6	..	100
588	Parent	47	None	1–1	2–2–1	25	20	76
588	F_1	45–47	None	3–2	2–4–8	62	60	39
588	F_2	45–47	None	5–5	9–4–2	87	21	78
588	F_3	45–47	None	2–2	3–2–3	38	45	55
588	F_1	45–47	Liv. ext.	3–3	1–3–1	52	6	90
588	F_2	45–47	Liv. ext.	4–4	5–2–1	38	5	92
588	F_3	45–47	Liv. ext.	..	.....	..	..	..
534	Parent	Stock	None	1–1	2–0–0	12	..	100
534	Parent	47	None	1–1	4–0–0	24	0	100
534	F_1	45–47	None	6–6	20–5–3	138	10	89
534	F_2	45–47	None	13–13	31–9–3	238	10	90
534	F_3	45–47	None	5–5	10–2–1	67	4	96
534	F_1	45–47	Liv. ext.	5–5	11–7–1	102	10	89
534	F_2	45–47	Liv. ext.	5–5	11–2–0	74	3	97
534	F_3	45–47	Liv. ext.	1–1	4–0–0	20	0	100

[1] Where both rations 45 and 47 are listed, the mothers were fed ration 47 during lactation and ration 45 at all other times. Ration 47 was ration 45 modified to contain 10% additional yeast in place of 10% dextrin.

[2] The first figure indicates the total number of mothers that had living young found at birth; the second figure, the number that had some young surviving to weaning.

[3] The first figure indicates the number of litters in which all young survived to weaning; the second figure, the number in which some young survived to weaning; the third figure, the number in which no young survived to weaning.

[4] Figures for stock ration include young born to mothers on stock ration during gestation but on ration 47 during lactation.

[5] Had no litters born on ration 47.

[6] The one female in this group died during the first mating.

vival being almost as good without liver extract as with this supplement in all generations. Another line (588), which in the parent generation exhibited slight impairment, dropped in the F_1 generation to 39% survival. The 467 line, in which all the young survived in the parent generation, dropped in the F_1 generation to 10% survival, all the survivors coming from the first litter of one rat. Both the 588 and 467 lines showed marked improvement in the F_2 and F_3 generations, the 467 line having an essentially normal survival record. It is evident, then, that there was in general a pronounced impairment in survival in the F_1 generation but decided improvement thereafter, with one line apparently able to resist impairment almost completely in all generations.

Several considerations may be involved in the improved record of the F_2 and F_3 generations. Since, of course, only the survivors could give rise to later generations, survival in these later generations would seemingly represent only that of the more resistant strains. Further, the intermingling of the 467 and 588 lines with the more resistant 534 line through cross-breeding may have made the later generations more resistant generally. That the intermingling of the 534 line with the other lines may have had some effect on survival is indicated by an examination, from the standpoint of the proportion of 534 ancestry involved, of the data on the matings of F_2 generation B_{12}-deficient mothers. Such an examination indicates that decreasing the percentage of 534 ancestry of F_3 generation young decreased the chances of their survival: 4 parts of 534, 100% survival; three parts, 97%; two parts, 88%; one part, 70%.

It is not clear, however, that the effect of 534 ancestry can be considered as truly hereditary. The resistance of certain animals to B_{12} deficiency, as judged by survival of the young, may possibly be explained on the basis of varying amounts of intestinal synthesis of vitamin B_{12}; thus, increasing the percentage of 534 ancestry may simply involve increasing the chances of transfer of the necessary bacteria. The post-weaning weight gains of the 534-line young in the earlier part

of the experiment, however, gave no indication of such synthesis. During the latter part of the experiment the weight gains of the F_3 and F_4 generation young did indicate the probability that such biosynthesis of B_{12}-active material was occurring in some of these animals, regardless of the line of descent involved. The incidence of such synthesis was no greater, however, than that observed among our regular test young put on B_{12}-deficient ration 47 at birth and placed at about 28 days of age on ration 45. It is evident, then, that it would be difficult to ascertain to what extent such synthesis may have affected the survival record of the young; but it can be said that all of the young that later showed a rather rapid rate of growth came from litters in which all the young had survived to weaning.

All mothers that received a liver extract supplement in this experiment had excellent records in regard to survival of their young, regardless of line or generation; not a single one carried less than 75% of its young to weaning.

It would appear from the results of this experiment that the effect of yeast may be primarily to delay rather than to prevent the drastic effects of B_{12} deficiency upon survival of young. Thus, while the record in the parent generation is more or less similar to that found in experiments 2 and 3, where the best results were observed on the yeast ration, yet in the F_1 generation the survival record for all except one resistant line approached more nearly that on the yeast-free rations.

This relatively better survival during the initial generation on yeast diets as compared to yeast-free rations was paralleled in some degree by a corresponding difference in birth weight (Dryden, Hartman and Cary, '51). This correlation is perhaps explained to some extent by the observations of Sica and Cerecedo ('48), who reported that on a stock diet as well as on a variety of experimental rations, the average birth weight of litters in which no young survived was lower than that of litters in which young survived, and that if the birth weight fell below a critical level, rats

usually failed to survive for more than a few days after birth. What factor there may be in yeast that could produce these differences in birth weight and survival of young between yeast-containing and yeast-free B_{12}-deficient rations is not clear. Such differences generally occurred to a much smaller extent on B_{12}-supplemented rations. That this factor could scarcely have been vitamin B_{12}-active material itself, in yeast, is indicated by rat growth assays in this laboratory, by microbiological assay of dried brewers' yeast (Peeler et al., '51), and by the failure of brewers' yeast to correct the poor survival of offspring of rats fed plant protein rations (Nell and Phillips, '50). That yeast could be promoting the synthesis of B_{12}-active material hardly seems probable; evidence reported elsewhere (Hartman, Dryden and Cary, '51) indicates rather that some of the yeast-free rations used here (diets 192, 195, 193, and 196) would have been much more likely to have promoted such synthesis. It is conceivable that there may be involved a deficiency of another factor, one that may possibly be synthesized when B_{12} is fed. Another possibility is that one effect of vitamin B_{12} in regard to survival of young might be in increasing the utilization of some other factor, which might be made available in larger quantities to the animal through the feeding of yeast.

Other workers using purified or semi-purified casein-containing rations have not always observed poor survival of newborn rats. Thus Schultze and Halvorson ('49) and Schultze ('50b) found no unusually high percentage of early deaths of young born to mothers on non-B_{12}-supplemented rations when these rations contained alcohol-extracted casein, although they did observe this condition when the rations contained commercial soy protein. Richardson and Russell ('51) reported that on a ration containing a commercial vitamin-test casein, survival to weaning was increased by adding B_{12} to the ration in the absence of added folic acid, but it is not clear from the data given that any material improvement resulted from adding B_{12} when folic acid was incorporated in

the ration. Mapson ('32, '33) observed that about 50 to 70% of the litters born to females on his rations died within the first three days of age; however, it is not clear to just what extent Mapson was dealing with a B_{12} deficiency, since he not only supplied some B_{12} to his test animals in the form of milk and probably in his "light white" casein but also found under his experimental conditions very little activity in Lilly 343 liver extract, which on the basis of the tests in this laboratory appears to be a very good source of vitamin B_{12}. Factors that have to be considered in interpreting all the above published results are the extent to which the ingredients of the ration were B_{12}-free and the possible bacterial synthesis of B_{12}-active material in the test animals.

Workers with rations composed primarily of cereal grains or vegetable seed products have usually obtained results more nearly similar to those reported in this paper for alcohol-extracted casein rations. Most of these investigators (Spitzer and Phillips, '46; Van Landingham and Lyon, '47; Heitman and Hogan, '49; Schultze and Halvorson, '49; Halvorson and Schultze, '50; Schultze, '49, '50b; Lepkovsky et al., '50, '51; Borson et al., '50; O'Dell, Whitley and Hogan, '51) have reported a large proportion of deaths during the first few days after birth of young rats born to mothers on rations of this type; the observed results have varied somewhat, depending to some extent upon the severity of the B_{12}-deficiency experienced and probably in some cases upon other deficiencies involved.

The situation is not altogether clear even on these vegetable protein rations, however. Thus Schultze ('50b) found that when commercial soy protein was subjected to alcohol extraction, early deaths of the newborn young no longer occurred to any great extent. Other workers using plant rations have failed to observe a high mortality of young prior to weaning (Watts et al., '50, with rats; Jaffé, '50, with mice) even though one of them (Jaffé) observed a growth response in the young to a single injection of crystalline B_{12}. These ob-

servations, together with our results on yeast-containing as compared to yeast-free rations, would seem to suggest that the early deaths of newborn young may be partially due to factors other than the existence of a B_{12} deficiency, even though the condition may be largely prevented or corrected by crystalline vitamin B_{12}.

SUMMARY

Results have been presented dealing with the survival of newborn rats on a number of B_{12}-deficient casein-containing rations. A very high percentage of deaths within the first few days after birth was found to occur on such rations when they contained crystalline B vitamins (except B_{12}) in place of yeast, and in some experiments when they contained yeast instead of the B vitamins. Some rats failed to show this phenomenon, the "resistance" in at least one line persisting through several generations.

Early deaths were not due to a failure of the mothers to secrete milk. Not only was the presence of considerable milk observed in the stomachs of the dead young, but in addition deficient mothers raised stock young while stock mothers generally failed to raise deficient young.

Vitamin B_{12} supplementation of the rations either prevented the occurrence of this phenomenon or restored survival to normal in most but not all cases; yeast-containing rations were somewhat but not greatly superior to yeast-free rations in this respect. No significant difference was found between the food consumption of B_{12}-deficient and B_{12}-supplemented mothers during gestation.

LITERATURE CITED

BORSON, H. J., D. SINGMAN, S. LEPKOVSKY, M. K. DIMICK, V. GASC AND R. PERRY 1950 Hematologic changes and death in vitamin B_{12}-deficient rats. Am. J. Physiol., *162:* 714.

CARY, C. A., AND A. M. HARTMAN 1948 Discussion on unidentified nutrient. Certified Milk, *23 (9):* 6.

DRYDEN, L. P., A. M. HARTMAN AND C. A. CARY 1949 The relation of vitamin B_{12} deficiency to ovarian function and reproduction in the rat. Abstr., 116th meeting, American Chemical Society, p. 39A.

———— 1951 The relation of vitamin B_{12} deficiency to fertility of the female and birth weight of the young in rats fed purified casein rations. J. Nutrition, *45:* 377.

HALVORSON, H. O., AND M. O. SCHULTZE 1950 Nutritional value of plant materials. III. Acute uremia of newborn rats from mothers fed plant rations. Ibid., *42:* 227.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 The role and sources of vitamin B_{12}. J. Am. Diet. Assn., *25:* 929.

———— 1951 The effect of riboflavin on the bacterial synthesis of vitamin B_{12}-active material in the rat. Arch. Biochem. Biophys., *34:* 324.

HARTMAN, A. M., L. P. DRYDEN, G. D. LIKELY AND C. A. CARY 1946 An unidentified nutrient in foods and feeds. J. Animal Sci., *5:* 404.

HEITMAN, H., JR., AND A. G. HOGAN 1949 Vitamin Deficiencies in Rations of Natural Feedstuffs. Missouri Agr. Sta., Research Bull. 432.

JAFFÉ, W. G. 1950 Reproduction of mice kept on rations low in vitamin B_{12}. Arch. Biochem., *27:* 464.

LEPKOVSKY, S., H. J. BORSON, R. BOUTHILET, R. PENCHARZ, D. SINGMAN, M. K. DIMICK AND R. ROBBINS 1951 Reproduction in vitamin B_{12}-deficient rats with emphasis upon intrauterine injury. Am. J. Physiol., *165:* 79.

LEPKOVSKY, S., H. J. BORSON, D. SINGMAN, R. PENCHARZ, R. BOUTHILET AND M. K. DIMICK 1950 The role of vitamin B_{12} in gestation, lactation and post-lactation in rats. Abstr., 117th meeting, American Chemical Society, p. 13A.

MAPSON, L. W. 1932 Evidence of the existence of a dietary principle stimulating general growth and lactation. Biochem. J., *26:* 970.

———— 1933 A deficiency disorder induced in suckling young rats bred on a purified synthetic diet with "Glaxo casein" (caseinogen) as sole source of protein. Ibid., *27:* 1061.

NELL, R. E., AND P. H. PHILLIPS 1950 Contributions of brewers' yeast to a diet deficient in reproductive factors. J. Nutrition, *42:* 117.

O'DELL, B. L., J. R. WHITLEY AND A. G. HOGAN 1951 Vitamin B_{12}, a factor in prevention of hydrocephalus in infant rats. Proc. Soc. Exp. Biol. Med., *76:* 349.

PEELER, H. T., H. YACOWITZ, C. W. CARLSON, R. F. MILLER, L. C. NORRIS AND G. F. HEUSER 1951 Studies on the vitamin B_{12} content of feedstuffs and other materials. J. Nutrition, *43:* 49.

RICHARDSON, L. R., AND E. L. RUSSELL 1951 Folic acid, crystalline vitamin B_{12} and penicillin in reproduction in rats. Fed. Proc., *10:* 391.

SCHULTZE, M. O. 1949 Nutritional value of plant materials. II. Prevention of acute uremia of the newborn rat by vitamin B_{12}. Proc. Soc. Exp. Biol. Med., *72:* 613.

———— 1950a Effect of phthalylsulfathiazole on growth and reproduction of rats fed a soybean protein ration. Ibid., *75:* 53.

SCHULTZE, M. O. 1950b Nutritional value of plant materials. V. Reproduction of rats fed purified rations containing soybean protein. J. Nutrition, *42:* 587.

SCHULTZE, M. O., AND H. O. HALVORSON 1949 Acute uremia of suckling rats born to mothers on rations devoid of animal protein. J. Animal Sci., *8:* 629.

SHILS, M. E., AND E. V. MCCOLLUM 1943 Further studies on the symptoms of manganese deficiency in the rat and mouse. J. Nutrition, *26:* 1.

SICA, A. J., AND L. R. CERECEDO 1948 Nutritional requirements of the rat for reproduction and lactation. Science, *107:* 222.

SPITZER, R. R., AND P. H. PHILLIPS 1946 Reproduction and lactation studies with rats fed natural rations. J. Nutrition, *32:* 631.

VAN LANDINGHAM, A. H., AND P. B. LYON 1947 Inadequacy of diets containing whole-grain cereals supplemented with soybean oil meal for lactation and growth of young rats. Arch. Biochem., *13:* 475.

WATTS, A. B., W. SWANK, R. J. OHMAN, O. B. ROSS AND R. W. MACVICAR 1950 The effect of supplementation of high corn diets on rat reproduction and lactation. J. Nutrition, *41:* 533.

STUDIES ON AMINO ACIDS IN SELF-SELECTED DIETS[1]

MARY F. FUTRELL, RUTH N. LUTZ, MAY S. REYNOLDS AND C. A. BAUMANN

Departments of Home Economics and Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication May 17, 1951)

This study is part of a cooperative project designed to determine the amount of the essential amino acids consumed by average women in the course of an ordinary day. The present experiments deal with the application of microbiological procedures to the determination of amino acids in self-selected diets. Four subjects in good health and 31 to 42 years of age consumed their usual meals at their respective homes, but for 30 days they weighed and recorded the amounts of all foods eaten. During two 4-day periods they also placed separate samples of all foods in waxed cardboard containers. These were brought to the laboratory daily, and appropriate aliquots were taken according to the amounts consumed. The foods were grouped in the following manner: "cereals," consisting of bread, cake and dry breakfast cereals; "food mixture," consisting of fruits, vegetables and prepared dishes that did not belong in the other categories; meats; eggs; cheese; and milk. All aliquots were stored in a refrigerator until the end of the 5-day period, when they were mixed in a Waring Blendor and frozen. The composition of all diets

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Contribution No. 1, Subproject 3, "Protein and amino acid requirements in human metabolism," of the North Central Region Cooperative Project NC-5, "Nutritional Status and Dietary Needs of Population Groups." Supported in part by funds 9B1 and 2 obtained under the Research and Marketing Act.

was calculated for 12 nutrients by means of a table of Donelson and Leichsenring ('45), and the diet preceding and following the balance periods was shown to be quite similar in composition to that eaten during the 10 days of the balance study (Lutz, '51).

Twenty-four-hour specimens of urine were collected during each 5-day period and preserved under toluene at 5°C. until analyzed for total nitrogen. Fecal samples were collected in an acetic-acid-alcohol mixture. The total collection was weighed, homogenized in a Waring Blendor and an aliquot preserved at 5°C. for nitrogen analysis.

All of the foods were thawed at the same time and aliquots weighed for direct analysis or for incorporation into a composite representing the entire intake during the experimental period. This composite was mixed in a Waring Blendor and 10 gm were then weighed for hydrolysis. The cereal mixture was further prepared for analysis by being dried at 60°C. in a vacuum oven, followed by thorough grinding in a mortar. Two grams of this powder were then hydrolyzed. Meat, eggs, or cheese were dried at 60° in a vacuum oven, followed by extraction with ether for 12 hours in a Goldfisch apparatus. Approximately 0.2 gm was used for hydrolysis. Ten grams of the milk sample and 20 gm of the food mixture were hydrolyzed in preparation for analysis.

Four food samples were weighed for each group of foods for both acid and alkaline hydrolysis. Two were used for duplicate analyses of the sample; one of the others contained an additional 0.2 gm of dried vitamin-free casein and the 4th sample, 0.2 gm of a "recovery mixture," consisting of the 10 essential amino acids. These were added for the purpose of determining the recovery of bound and free amino acids respectively in the presence of the various food materials. Furthermore, 0.2 gm of casein and 0.2 gm of the recovery mixture were hydrolyzed and themselves analyzed for the various amino acids in the absence of any additional foods. The samples were hydrolyzed for 8 hours at 15 pounds pressure in 4 N HCl; for determination of tryptophan the

samples were autoclaved with 4 N NaOH for 15 hours. After autoclaving, the samples were filtered, adjusted to pH 6.8, and diluted to 100 ml. These were further diluted for assay as needed.

The hydrolysates were analyzed for 10 amino acids by the microbiological methods of Steele et al. ('49). The assay organisms used were *Leuconostoc mesenteroides* P-60 for all amino acids except valine and methionine, *Lactobacillus delbruckii* for valine, and *Leuconostoc citrovorum* 8081 for methionine. Several of the amino acids were determined with both *L. mesenteroides* and *L. citrovorum*. The total nitrogen of urine, feces, and food samples was determined by the method of Scales and Harrison ('20).

RESULTS

Validity of methods

The most satisfactory recoveries of free or bound amino acids were observed in the presence of high-protein foods such as meat, in which they ranged from 93 to 98.5% (table 1). Recoveries from milk, cheese and eggs were very similar to those observed with meat. On the other hand, unsatisfactory recoveries were obtained for certain of the amino acids added to cereals or crude food mixtures: e.g., 80 to 84% for tryptophan, 80 to 85% for lysine, and 81 to 87% for histidine. The recoveries for methionine were also low with a somewhat greater variability, depending upon the nature of the food material present. In the presence of cereals the recoveries ranged from 86 to 90%, while in the presence of fruits and vegetables, the so-called "food mixture," or the entire food composite, recoveries ranged from 80 to 85%. Certain of the amino acids yielded satisfactory recoveries even in the presence of the cereals or other low-protein foods. These were isoleucine, 92 to 96%; leucine, 91 to 95%; and valine 92.5 to 96%. The other amino acids yielded intermediate recoveries.

The percentage recoveries were usually better when the amino acids were added in a bound form, as casein, than as

a mixture of free amino acids. The discrepancies between the two were never large but were observed consistently for all 10 amino acids in the presence of each of the cruder food materials (table 1). This discrepancy is probably due to the fact that destruction of the free amino acid begins immediately in the presence of carbohydrate during hydrolysis. Since the amino acids in food are mainly in the bound form, the true recoveries probably lie closer to those for casein than to those for the free amino acids.

Since the recoveries for many of the amino acids in the cereal and food mixtures were less than 100%, it is apparent that the values obtained by microbiological analysis of the hydrolysate are somewhat less than the true values. Fortunately, however, the poorer recoveries were observed in the low-protein foods, and hence the error in the determination did not greatly affect the values for the total amount consumed during the day.

Table 2 illustrates the daily lysine and methionine consumption by 4 subjects during two dietary periods as determined by various methods. The lowest values were invariably those determined directly on a representative composite of all the foods consumed during that period. Significantly higher figures were obtained when the various types of foods were analyzed separately and the results totaled (table 2, column B). Since others (Mertz et al., '52) have determined amino acids on entire composites, factors have been calculated for each amino acid indicating the correction to be applied to such values. These latter factors involved the total amount in the diet as obtained by separate analyses, divided by the results of determinations made directly on composites (table 2, column C). The factor equals B divided by A, and for the 10 amino acids (table 3) ranged from 1.04 for isoleucine to 1.5 for methionine (1.3 to 1.7). The highest factor, 1.7, was for a diet that contained very little high-protein food; the factor 1.3, for a diet that was made up largely of high-protein foods. Thus, if a composite sample contains large amounts of high-protein foods and less carbo-

TABLE 1

Percentage recoveries of free and bound amino acids added to various foods

AMINO ACID	MEAT [1]		CEREALS		FOOD MIXTURE		FOOD COMPOSITES	
	Casein	Amino acids	Casein	Amino acids	Casein	Amino acids	Casein	Amino acids
	%	%	%	%	%	%	%	%
Arginine	96.5	95.0	92.0	87.5	95.0	90.5	93.5	88.0
Histidine	97.0	96.5	87.0	84.0	85.0	81.0	85.0	82.0
Isoleucine	98.5	97.5	94.0	92.0	96.0	93.0	95.0	92.5
Leucine	98.5	98.0	94.0	91.0	95.0	93.0	95.0	93.0
Lysine	97.0	95.5	85.5	82.0	85.0	80.0	85.0	80.5
Methionine	97.5	94.0	90.0	86.0	85.0	80.0	84.0	81.0
Phenylalanine	96.0	93.5	94.0	91.0	88.0	84.0	89.5	85.0
Threonine	98.0	96.5	93.5	90.5	94.0	90.0	94.5	91.0
Tryptophan	96.0	93.0	84.0	81.5	83.0	90.0	84.0	81.5
Valine	98.5	98.0	94.5	92.5	96.0	93.5	96.0	93.5

[1] Recoveries from milk, cheese, and eggs were similar to those for meat.

hydrate, the correction factor would be lower than the average factor, while for a composite high in carbohydrate and low in protein, the correction factor would be greater than the average. Table 3 also shows that the correction factors for isoleucine, leucine, and valine are virtually negligible.

TABLE 2

Calculation of factors for correcting results obtained on composites

AMINO ACID		A Determination on composite	B Totals	C Factor B/A
		gm	*gm*	
	Lysine			
G.N.	I_1	3.94	4.95	1.26
	I_2	3.00	3.63	1.21
Z.W.	II_1	5.60	6.17	1.10
	II_2	5.00	5.75	1.15
E.J.	III_1	3.85	5.75	1.50
	III_2	3.13	4.69	1.50
C.W.	IV_1	5.15	7.17	1.39
	IV_2	4.25	6.31	1.48
Average				1.3
	Methionine			
G.N.	I_1	0.85	1.14	1.4
	I_2	0.54	0.90	1.7
Z.W.	II_1	0.92	1.39	1.5
	II_2	0.90	1.35	1.5
E.J.	III_1	1.36	2.02	1.5
	III_2	1.34	1.90	1.5
C.W.	IV_1	2.02	2.54	1.3
	IV_2	1.82	2.28	1.3
Average				1.5

For comparison with the data of others (Patton et al., '48; Friedman and Kline, '50) on amino acid losses incurred by heating with reducing carbohydrates table 4 was compiled, by assuming that the values for the totals obtained by analyzing separate foods ("B" of table 2) were correct, and that the results of analyses on food composites were comparable to those obtained by others who heated proteins or

amino acids with glucose. The percentages of the various amino acids "lost" in the analysis of entire composites were found to vary markedly for the different amino acids. Losses for isoleucine, leucine, and valine were almost negligible, whereas for tryptophan, methionine and lysine they ranged from 24 to 30%. In general these differences between amino acids were quite similar both quantitatively and qualitatively to those observed by Patton et al. ('48) and Friedman and Kline ('50).

TABLE 3

Factors for correcting results obtained on composites

AMINO ACID	AVERAGE	RANGE
Arginine	1.18	1.07–1.27
Histidine	1.26	1.16–1.37
Isoleucine	1.04	1.02–1.07
Leucine	1.05	1.04–1.06
Lysine	1.30	1.15–1.50
Methionine	1.50	1.30–1.70
Phenylalanine	1.15	1.13–1.17
Threonine	1.12	1.11–1.14
Tryptophan	1.47	1.24–1.77
Valine	1.06	1.04–1.07

Amino acid content of diets

The total daily intakes of 10 amino acids consumed by the 4 subjects during each of two periods are presented in table 5, together with the intake of protein and the nitrogen balance. Two of the subjects were found to be in negative nitrogen balance during each of the two periods, while the other two subjects remained in positive balance. The amounts of protein consumed by the subjects ranged from 50 to 94 gm per day. At these levels of protein intake the consumption of most of the essential amino acids was well above the levels of intake tentatively recommended by Rose ('49). As a typical example, the consumption of isoleucine by our subjects ranged from 2.5 to 5.8 gm per day, compared with a recommended level of 1.4 gm and a minimum level of only

0.7. The daily intakes of leucine, lysine, threonine and valine were likewise at least double the recommended levels and 4 to 6 times the minimum levels (table 5). In most of the diets phenylalanine and tryptophan were present in amounts above the recommended level, but the factor of safety was not large except in two diets that supplied 87 to 94 gm of protein per day.

Compared with Rose's "tentative recommended" levels of intake, the limiting amino acid in all diets appeared to be methionine. The amounts of this amino acid consumed by

TABLE 4

Discrepancy between results on composites vs. separate foods compared with losses of amino acids when heated with carbohydrates

AMINO ACID	DISCREPANCY IN PRESENT VALUES	FRIEDMAN AND KLINE, '50	PATTON, HILL AND FOREMAN, '48
	% Loss	% Loss	% Loss
Arginine	15	12	33
Histidine	20	29	7
Isoleucine	5	5	6
Leucine	5	7	3
Lysine	24	22	26
Methionine	28	18	
Phenylalanine	14	22	
Threonine	12	29	
Tryptophan	30	22	39
Valine	5	9	

our subjects were never much above the recommended level, even in a diet that supplied 94 gm of protein per day (diet IV, table 5). Only two of the 8 diets analyzed contained more than the recommended 2.2 gm of methionine daily; 5 contained amounts ranging between the minimum and the recommended level, and one was below the minimum necessary for maintenance. All of the subjects that were in negative nitrogen balance were receiving less than Rose's "tentative recommended" amount of methionine. This latter recommendation, however, was based on experiments with a synthetic diet that contained no cystine (Rose, '49; Rose et al.,

TABLE 5

Daily consumption of amino acids and protein by women

(Totals of analyses on separate foods)

SUBJECT		PROTEIN	ARGININE	HISTIDINE	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYLALANINE	THREONINE	TRYPTOPHAN	VALINE	NITROGEN BALANCE
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm N/day*
G.N.	I	52	2.58	1.41	3.06	3.80	4.95	1.14	2.44	2.10	0.58	4.10	— 0.5
	I_2	50	2.13	1.26	2.49	3.28	3.63	0.90	1.98	1.68	0.50	3.19	—1.09
Z.W.	II_1	59	3.46	1.97	3.75	5.43	6.17	1.39	2.87	2.50	0.69	5.39	+ 0.5
	II_2	58	3.05	1.81	3.57	4.90	5.75	1.35	2.66	2.34	0.68	4.51	+ 0.7
E.J.	III_1	62	3.75	2.43	4.74	5.77	5.75	2.02	3.22	2.91	0.76	3.28	— 0.1
	III_2	59	3.03	1.91	4.19	4.97	4.69	1.90	2.80	2.41	0.65	2.85	— 0.3
C.W.	IV_1	94	5.26	3.00	5.73	7.35	7.17	2.54	4.88	3.44	1.28	4.58	+ 3.8
	IV_2	87	4.52	2.62	5.41	6.34	6.31	2.28	4.25	3.14	1.01	3.97	+ 1.3
Recommended intake[1]					1.4	2.2	1.6	2.2	2.2	1.0	0.50	1.6	
Minimum intake[1]					0.7	1.1	0.8	1.1	1.1	0.5	0.25	0.8	

[1] Rose, Fed. Proc., *8:* 546, 1949.

Physical data pertaining to subjects in above table

SUBJECT	AGE	HEIGHT	WEIGHT	DAILY CALORIC INTAKE
	years	*inches*	*lb.*	
G.N.	31	66½	145	1,590
Z.W.	40	66½	112.5	1,670
E.J.	34	61½	115	1,800
C.W.	42	64	119	2,000

'50), and it probably does not apply to natural cystine-containing diets such as our subjects were consuming.

The parallelism between nitrogen balance and methionine intake was not absolute. Subject II consumed 1.4 gm of methionine per day and remained in positive nitrogen balance, whereas subject III was in negative nitrogen balance on 1.9 to 2.0 gm of methionine per day. All of the other essential amino acids appeared to be in excess in this latter diet. It is therefore possible that subject III was in negative N balance because she was not utilizing her methionine or other limiting amino acids optimally, possibly because of unequal distribution of proteinaceous foods throughout the day. The breakfast of subject III consisted of coffee and toast during both experimental periods, whereas the breakfast of subject II, who remained in positive nitrogen balance on only 1.4 gm of methionine per day, consisted of milk and eggs besides the usual toast and coffee. The two subjects weighed 112.5 and 115 pounds, respectively, and therefore their requirements must have been similar.

The present diets furnished 2.2 to 5.3 gm of arginine per day and 1.3 to 3.0 gm of histidine. Since it has been difficult to demonstrate a requirement of the human adult for these amino acids (Rose et al., '51), it is likely that the amounts present were well above those actually needed.

DISCUSSION

The determination of methionine is less satisfactory than the determinations for most of the other essential amino acids, and only lysine and tryptophan are subject to greater losses on hydrolysis in the presence of reducible carbohydrate (Friedman and Kline, '50). Nevertheless, in the diets analyzed the values obtained for the amounts of these latter two amino acids, like the values for leucine or valine, which are easily determined, were well above the levels recommended by Rose ('49), whereas methionine was consistently low.

An interpretation of this latter finding, however, must await determination of the amount of methionine needed by human subjects fed a purified diet containing adequate amounts of cystine. If man is like the rat in that only one-sixth of the total methionine requirement can be met by cystine (Womack and Rose, '41; Rose et al., '48), the application of Rose's "strictly tentative" methionine requirement to the diets of our subjects would still leave methionine the limiting amino acid in all diets. But if cystine should prove to have a relatively large sparing effect on the methionine requirement of man, the amounts of methionine in our diets might prove to be more than adequate and the negative nitrogen balance to be due to a limitation in some other amino acid. In this connection it is of interest that the only other amino acid in our diets which was below Rose's recommended levels was phenylalanine in diet I_2 (table 5), another essential amino acid that serves as a specific precursor for a non-essential one missing from Rose's assay diet.

The present data support the suggestion that the maintenance of nitrogen balance may depend on the amounts of animal protein consumed at different periods of the day (Leverton and Gram, '49), and it would appear that a survey of amino acid intakes by segments of the population should include the amounts of the critical amino acids consumed at separate meals. Controlled experiments on subjects consuming equal amounts of methionine but with these amounts distributed unequally throughout the day should also yield interesting results.

SUMMARY

1. Ten essential amino acids were determined microbiologically in the self-selected diets of 4 women. Determinations were made both on a composite sample and on separate groups of foods.

2. The total intake of amino acids always appeared to be greater when determined on separate foods than on composites. The greatest discrepancies were found with lysine, methionine and tryptophan, with intermediate losses observed

for histidine, threonine, phenylalanine and arginine. Values for leucine, isoleucine and valine were similar whether made on the separate foods or on the composites.

3. Satisfactory recoveries of bound and free amino acids were found in all cases in the presence of high-protein foods but in the presence of carbohydrate-rich foods the losses ranged as indicated above.

4. The amounts of most of the essential amino acids consumed by the subjects were significantly higher than the levels recommended by Rose. Methionine, however, appeared to be limiting. Negative nitrogen balance seemed to be related to total protein intake, and also to the distribution of protein in meals throughout the day.

LITERATURE CITED

DONELSON, E. G., AND J. M. LEICHSENRING 1945 Food composition tables for short method of dietary analysis. J. Am. Diet. Assn., *21:* 440–442.

FRIEDMAN, LEO, AND O. L. KLINE 1950 The relation of the amino acid-sugar reaction to the nutritive value of protein hydrolysates. J. Nutrition, *40:* 295.

LEVERTON, RUTH, AND MARY GRAM 1949 Nitrogen excretion of women related to the distribution of animal protein in daily meals. Ibid., *39:* 57.

LUTZ, RUTH 1951 Studies of the dietary practices and the metabolism of protein and certain of the B vitamins in mature women on self-selected diets. M.S. thesis, University of Wisconsin.

MERTZ, E. T., J. BAXTER, L. JACKSON, C. E. RODERUCK AND A. WEIS 1952 Essential amino acids in self-selected diets of older women. J. Nutrition, *46:* 313.

PATTON, A. R., E. G. HILL AND E. M. FOREMAN 1948 Amino acid impairment in casein heated with glucose. Science, *107:* 623.

ROSE, W. C. 1949 Amino acid requirements of man. Fed. Proc., *8:* 546.

ROSE, W. C., W. J. HAINES, D. T. WARNER AND J. E. JOHNSON 1951 The amino acid requirements of man. II. The role of threonine and histidine. J. Biol. Chem., *188:* 49.

ROSE, W. C., J. E. JOHNSON AND W. J. HAINES 1950 The amino acid requirements of man. I. The role of valine and methionine. Ibid., *182:* 541.

ROSE, W. C., M. J. OESTERLING AND M. WOMACK 1948 Comparative growth on diets containing ten and nineteen amino acids, with further observations upon the role of glutamic and aspartic acids. Ibid., *176:* 753.

SCALES, F. M., AND A. P. HARRISON 1920 Boric acid modification of the Kjeldahl method for crop and soil analysis. Ind. Eng. Chem., *12:* 350.

STEELE, B. F., H. E. SAUBERLICH, M. S. REYNOLDS AND C. A. BAUMANN 1949 Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. J. Biol. Chem., *177:* 533.

WOMACK, M., AND W. C. ROSE 1941 The partial replacement of dietary methionine by cystine for purposes of growth. Ibid., *141:* 375.

ESSENTIAL AMINO ACIDS IN SELF-SELECTED DIETS OF OLDER WOMEN[1]

E. T. MERTZ,[2] E. J. BAXTER,[3] L. E. JACKSON,[4] C. E. RODERUCK[5] AND A. WEIS[6]

Agricultural Experiment Stations of Indiana, Michigan, Iowa and Missouri

(Received for publication August 9, 1951)

In a cooperative study of the nutritional status and dietary habits of older women, it was revealed that a large proportion of the subjects on self-chosen diets exhibited negative nitrogen balance when intakes approached, or even exceeded, the present recommended dietary allowances for protein (unpublished data, North Central Region Cooperative Project NC-5). Determination of daily intakes of the essential amino acids therefore appeared desirable, and representative food samples were collected from subjects on self-chosen diets. At Michigan State College, Iowa State College, and the University of Missouri, aliquots of these samples were stored in hydrochloric acid and shipped to Purdue University, where they were analyzed by microbiological procedures for 7 of the 8 amino acids[7] considered essential for nitrogen balance in human adults. The results of these analyses are presented here.

[1] Contribution 2, Subproject 3, "Protein and Amino Acid Requirements in Human Metabolism," of the North Central Region Cooperative Project NC-5, "Nutritional Status and Dietary Needs of Population Groups."

[2] Journal paper 554, Purdue Agricultural Experiment Station, Agricultural Chemistry Department, Lafayette, Ind.

[3] See footnote 2.

[4] Journal paper 1271, Michigan Agricultural Experiment Station, Foods and Nutrition Department, E. Lansing.

[5] Journal paper J-1971, Iowa Agricultural Experiment Station, Foods and Nutrition Department, Ames. Project No. 1028.

[6] Journal paper 1264, Missouri Agricultural Experiment Station, Foods and Nutrition Department, Columbia.

[7] Tryptophan could not be determined in the composites inasmuch as it was destroyed by the HCl during storage.

EXPERIMENTAL

1. Preparation of the food composites

The 18 women who served as subjects in the balance experiments were in the age range of 33 to 77 years and represented groups of different economic and social levels (Ohlson et al., '50). Each day of a 5-day balance period (or two consecutive 5-day balance periods) the subject collected: (a) weighed aliquots of the total food consumption; (b) the total fecal output; and (c) the total urine excretion. Nutrient liquids and nutrient solids were preserved with 200 ml of concentrated HCl and 500 ml of 20% HCl, respectively, over the 5-day period.

The preparation of the liquid and solid food digests was patterned after that of Stearns ('29) for fecal digests. Records of food consumed during the 10 days prior to the balance period indicated no significant changes in food habits during the balance period. Aliquots of the liquid and solid composites were shipped to Purdue. The acid composites as received were brown in color and varied from 0.5 to 3.6 N when titrated with alkali.

2. Amino acid analyses

The food composites were hydrolyzed with 10% HCl for 10 hours in sealed ampoules in an autoclave at 15 pounds of steam pressure and analyzed using the microbiological methods of Dunn et al. ('49). A sample of casein of known amino acid content was hydrolyzed and analyzed with each group of food composites.

The cooperating laboratories provided the data on the total volume of food composite collected and its relation to the average total amount of food ingested by the subject in 24 hours. From this the average daily intakes of the various amino acids were calculated. Nitrogen balance data and calculated caloric intakes were supplied by the cooperating laboratories.

In parallel studies, Futrell, Lutz, Reynolds and Baumann ('52) compared the apparent amino acid composition of a day's meals when the foods were mixed together and hydrolyzed, and when the individual foods were hydrolyzed separately. They found that a partial destruction of each of the essential amino acids appeared to occur in the food mixture during acid hydrolysis. The extent of destruction varied with the nature of the foods in the composite, being lowest for the amino acids in composites containing large amounts of meat, eggs, and cheese, and highest for the amino acids in composites containing large amounts of cereals.

The greatest amount of destruction occurred with the amino acids methionine, tryptophan and lysine. Thus, the correction factor required for methionine in meat, eggs and cheese was 1.30; for methionine in cereal mixtures, 1.70. For a composite of the day's meals it was suggested that an average factor, 1.50, could be used for methionine. On the same basis, the average correction factors proposed for tryptophan and lysine in mixed composites were 1.47 and 1.30, respectively. The average correction factors proposed for the remaining essential amino acids were as follows: isoleucine, 1.04; leucine, 1.05; phenylalanine, 1.15; threonine, 1.12; and valine, 1.06.

We have used the average correction factors proposed by the Wisconsin group in calculating the daily amino acid intakes of our subjects.

RESULTS AND DISCUSSION

The physical characteristics of the subjects and data with respect to their selection of food are summarized in table 1. One-half of the subjects were in the 5th decade of life. They varied in build and activity, and in their selection of food. Many of the subjects consumed a fairly adequate diet for two of the three meals, then either omitted entirely or consumed a very unbalanced third meal.

The average daily intakes of calories, crude protein and amino acids are summarized in table 2. Each period designated in table 2 is a 5-day period, and the average daily in-

TABLE 1

Physical characteristics and food selection

SUBJECT	AGE	WT.	HT.	BUILD	ACTIVITY	FOOD SELECTION
	yr.	*kg*	*cm*			
Go	68	63	155	Average	Active, nervous	Good.
P	60	62	152	Obese	Active	Very little meat or meat substitute (except eggs).
J	77	74	173	Tall	Unusually active	Good except little milk and eggs.
B	56	77	166	Heavy	Moderate, some activity	Good.
E	77	69	165	Average	Unusually active	Well balanced, but low intake.
F	59	69	161	Heavy	Active, vigorous	Low vegetable intake.
Gr	59	62	148	Medium	Hard worker, tired	Low intake of milk, meat, vegetables.
Ch	52	74	158	Small-fat	Sedentary	Fair selection. Some source of animal protein at nearly every meal. Low intake of fruit and vegetables.
K	41	55	156	Short-medium	Moderate	Poor. No protein food at breakfast, low on fruits.
R	58	91	174	Heavy, big-boned	Moderate	Good.
Cr	70	55	160	Slender-medium	Moderate	Poor distribution of protein foods between meals. Narrow selection of fruits and vegetables.
E	33	52	164	Thin, small-boned	Very active	Fair. Little animal protein at breakfast.
V	71	65	163	Medium	Sedentary	Poor (similar to that of subject Cr).
MGM	57	55	156	Stocky	Average	Poor. No protein at breakfast, no milk.
GMC	57	72	166	Stocky	Very active	Poor. Fruit juice constituted breakfast. Other meals, fair selection.
RED	64	55	168	Slender	Active	Good breakfast and lunch, poor dinner.
LS	57	62	163	Average	Average	Very poor. Period 1 — no lunch 1 day, no protein food at dinner 4 days. Period 2 — no dinner 2 days, inadequate dinner 3 days (example, doughnut and coffee, tomatoes and coffee).
SK	52	72	160	Stocky	Active	Good breakfast and dinners. Poor lunch (period 1): no protein food, often only dessert.

take of each nutrient is based on analysis of one liquid and one solid food composite representing the food eaten during the 5-day period. Average (calculated) daily intakes of calories varied from 850 (subject GMC-1) to 2,288 (subject MGM-2). Average daily intakes of crude protein ($N \times 6.25$) varied from 20.9 gm (subject LS-1) to 92.6 gm (subject R). About two-thirds of the subjects shown in table 2 were in negative nitrogen balance. In the major metabolism study, however, the number of subjects in negative nitrogen balance and the number in positive nitrogen balance were about equal (unpublished data).

The minimum daily intake of each amino acid required to maintain older women in nitrogen balance is not known. Information is available only on the minimum requirements of young adult males on purified diets containing mixtures of 8 amino acids (those in table 2 plus tryptophan). In these experiments, Rose ('49) found that the minimum daily intakes of each amino acid required for nitrogen balance were in the range of 0.5 to 1.1 gm. These minimum intakes are probably different from the minimum daily intakes required for nitrogen balance when ordinary foods are eaten, for Rose has pointed out that an unusually large number of calories are needed to secure nitrogen balance when free amino acids are the sole source of dietary nitrogen. Although the caloric requirements may be reduced when intact proteins are ingested, nevertheless many self-selected diets appear to be low in calories (see table 2). Furthermore, the cost of digestion must be taken into account in assessing the value of the intact proteins in self-chosen diets, and it must be recognized that the amino acid mixture of Rose was of constant composition, whereas the amino acid pattern in ingested natural foods varies from meal to meal. Finally, Rose fed glycine or urea as an extra source of ammonia nitrogen to take the place of the natural non-essential amino acids. Had cystine and tyrosine furnished part of the nitrogen, it is possible that the requirements for methionine and phenylalanine might have been reduced.

TABLE 2

Average daily intake of calories, protein and amino acids[1] by older women

STATE	SUBJECT (Initials-periods)	CALORIES	PROTEIN	NITROGEN RETENTION	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYL-ALANINE	THREONINE	VALINE
			gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Mich.	Go-1C	1,535	53.7	+ 0.18	2.6	3.8	2.8	1.0 (1.2)	2.5	1.5	3.3
	P -1B	1,094	32.3	— 2.44	2.0	2.7	1.9	1.1 (1.2)	2.5	1.1	2.7
	J -1A	1,478	91.8	+ 4.19	3.0	4.5	3.8	1.6 (1.8)	2.6	1.7	3.4
	B -1A	1,930	71.3	+ 1.20	3.9	6.2	6.0	2.4 (2.7)	3.5	2.7	4.1
	B -1B	1,807	75.1	+ 1.25	3.9	5.8	4.7	1.7 (2.0)	3.3	3.0	4.1
	E -1C	1,656	47.7	— 0.48	3.2	4.6	3.8	1.7 (1.9)	3.3	2.2	3.7
	E -1D	1,630	60.4	+ 0.50	2.0	3.7	2.8	1.3 (1.5)	1.9	1.5	2.6
	F -1A	1,765	68.0	— 0.62	3.0	5.5	5.6	2.4 (2.7)	3.4	2.5	3.8
	F -1B	1,489	52.5	— 1.71	2.6	3.9	3.6	1.3 (1.5)	2.2	1.8	2.9
	Gr-1A	883	32.8	— 1.67	1.3	2.1	3.1	0.6 (0.7)	1.1	1.0	1.5
	Gr-1B	1,185	36.3	— 1.73	1.6	2.4	3.4	0.8 (0.9)	1.4	1.0	1.8
Iowa	Ch	2,101	81.2	+ 1.05	3.9	5.7	5.5	1.8 (2.0)	3.7	1.5	4.6
	K	1,278	48.5	+ 0.28	2.4	3.1	3.3	0.9 (1.0)	1.9	0.9	2.5
	R	1,820	92.6	+ 0.41	4.5	6.2	7.3	1.9 (2.2)	3.6	1.8	4.9
	Cr	1,169	34.1	— 1.53	1.3	3.9	1.8	1.3 (1.4)	1.4	1.1	1.5
	E	1,911	61.4	— 1.61	2.6	7.8	3.8	2.8 (3.2)	2.5	2.2	2.9
	V	1,299	38.8	— 2.25	1.5	4.6	2.8	1.4 (1.6)	1.4	1.2	1.6
Mo.	MGM-1	1,774	47.5	— 1.95	1.6	2.8	4.0	1.0 (1.2)	1.9	2.5 [2]	2.0
	MGM-2	2,288	66.9	— 0.63	2.4	4.0	5.2	1.6 (1.8)	2.7	3.8 [2]	3.0
	GMC-1	850	43.5	— 2.37	1.0	1.8	2.5	0.8 (0.9)	1.1	1.6 [2]	1.3
	GMC-2	1,128	34.8	— 2.93	1.2	2.1	2.8	0.8 (0.9)	1.3	1.7 [2]	1.5
	RED-1	1,465	59.8	— 0.62	2.4	4.0	5.2	1.5 (1.8)	2.8	3.0 [2]	2.9
	RED-2	1,850	61.0	— 1.13	2.2	3.9	5.3	1.9 (2.2)	2.9	3.0 [2]	2.7
	LS-1	1,208	20.9	— 2.50	0.8	1.4	1.4	0.5 (0.6)	1.0	1.1 [2]	0.9
	LS-2	959	21.5	— 5.47	0.7	1.3	1.3	0.4 (0.5)	0.9	1.0 [2]	0.8
	SK-1	1,902	59.6	— 1.56	2.1	4.0	5.1	1.6 (1.8)	2.5	2.9 [2]	2.8
	SK-2	1,742	54.7	— 1.59	2.3	4.2	5.1	1.4 (1.6)	2.6	3.4 [2]	2.9

[1] The amino acid values have been corrected for losses during acid hydrolysis of the composite by multiplying our experimental values by the average correction factors listed in the text. The methionine values in parentheses were obtained by multiplying our experimental values by the factor 1.7, which would correct for the maximum losses obtained during acid hydrolysis by Futrell et al. ('52; i.e., the hydrolysis of composites rich in cereals). The corrected values in the second decimal place were dropped if below 0.05, and added as 0.1 if 0.05 or above.

[2] Determined by the microbiological method of Steele et al. ('49).

With the above limitations in mind, it is of interest to compare the average daily amino acid intakes of older women on self-chosen diets with the minimum and recommended intakes proposed by Rose as "strictly tentative" for young adult males (table 3). When compared in this manner, methionine appears to be the first limiting amino acid in the self-chosen diets of older women. Even after applying the *least favorable* correction factor (which would assume a diet composed only of cereal foods), out of 27 balance periods only 5 showed a level of daily methionine intake equal to or higher

TABLE 3

Comparison of amino acid intakes in this study with recommended intakes for adult males[1]

	ISO-LEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYLALANINE	THREONINE	VALINE
(A) Recommended intake (gm)	1.4	2.2	1.6	2.2	2.2	1.0	1.6
(B) Minimum requirement (gm)	0.7	1.1	0.8	1.1	1.1	0.5	0.8
Number in table 2 at or above (A)	21	22	25	3(5)[2]	16	26	21
Number in table 2 at or below (B)	1	0	0	10(7)	4	0	1

[1] Rose ('49).

[2] The numbers in parentheses are based on "corrected" values obtained by using the factor 1.7 (see table 2).

than the level recommended by Rose, and in 7 out of 27 periods the methionine intake fell below the minimum level determined with purified diets.

Whether part of the methionine requirement was met by the cystine in the diet is not known. If adult humans are no more efficient than young rats in this substitution, in many instances cystine would not have been able to correct the deficiency. Womack and Rose ('41) have shown that in young rats only one-sixth of the methionine requirement may be satisfied by the dietary addition of cystine. However, in recent experiments with chicks (West, Carrick, Hauge and

[1] Determined by the microbiological method of Steele et al. ('49).

Mertz, '51), cystine supplied two-thirds of the total sulfur amino acid requirements.

Inspection of table 3 indicates that phenylalanine may be the next limiting amino acid in self-selected diets of older women. Here again, part of the phenylalanine requirement may be met by tyrosine. In young rats (Womack and Rose, '46) tyrosine can replace about one-half of the phenylalanine needed in the diet.

In experiments parallel to the ones reported here and relating to 4 women living on self-selected diets, Futrell et al. ('52) determined the average daily intakes of the 7 amino acids listed in table 2 and, in addition, determined the intakes of tryptophan. They found that the amounts of most of the essential amino acids consumed by their 4 subjects were higher than the levels recommended by Rose. Here again, however, methionine appeared to be limiting.

The studies presented here raise several questions concerning the adequacy of recommended protein intakes when considered in relation to the habits of food selection and the distribution of food in the meals consumed by a group of aging women. That a low intake of calories and an irregular ingestion of high quality protein at each of the three meals may contribute toward the high incidence of negative retention of nitrogen has been suggested. To counterbalance this, it is quite possible that the amino acid requirements of aging women are somewhat lower than those of young men. On the average, women are 15% smaller than men. If it is accepted that the requirement per unit weight is the same for both sexes, women should require correspondingly less of each amino acid. When the recommended allowances of Rose are used as criteria, there is low consumption of methionine in comparison to that of the other amino acids studied. This in turn would indicate a poor selection of foods by aging women (see table 1).

The studies reported here comprise a preliminary survey, and have helped to point up several areas in which research needs to be done before an accurate assessment of the ade-

quacy of the amount of protein in the diet of aging subjects can be made. Thus, the quantitative relationship between caloric intake and minimum requirements for the essential amino acids needs to be investigated, the intake of individual amino acids at each meal should be correlated with nitrogen balance, and the replacement value of cystine for methionine and tyrosine for phenylalanine should be determined. The data already obtained suggest that self-selected diets of older women could be improved by the addition of foods rich in methionine.

SUMMARY

With the use of microbiological methods, the average daily intakes of 7 essential amino acids were determined for 18 older women on self-selected diets. The levels of intake of isoleucine, leucine, lysine, threonine, and valine usually equaled or exceeded the recommended levels of Rose, whereas phenylalanine was intermediate and methionine intake seldom reached the recommended levels and in many cases was below the minimum level. Methionine appears to be the first limiting essential amino acid in the self-selected diets studied.

LITERATURE CITED

DUNN, M. S., M. N. CAMIEN, R. B. MALIN, E. A. MURPHY AND P. J. REINER 1949 Percentages of twelve amino acids in blood, carcass, heart, kidney, liver, muscle, and skin of eight animals. Univ. of Calif. Publ. in Physiol., *8:* 293.

FUTRELL, M. R., R. LUTZ, M. S. REYNOLDS AND C. A. BAUMANN 1952 Intake of the essential amino acids by adult women on self-selected diets. J. Nutrition, *46:* 299.

OHLSON, M. A., L. JACKSON, J. BOEK, D. C. CEDERQUIST AND W. D. BREWER 1950 Nutrition and dietary habits of aging women. Am. J. Public Health, *40:* 1101.

ROSE, W. C. 1949 Amino acid requirements of man. Fed. Proc., *8:* 546.

STEARNS, G. 1929 A rapid method for the preparation of fecal digests suitable for use in nitrogen and mineral analyses. J. Lab. Clin. Med., *14:* 954.

STEELE, B. F., H. E. SAUBERLICH, M. S. REYNOLDS AND C. A. BAUMANN 1949 Media for *leuconostoc mesenteroides* P-60 and *leuconostoc citrovorum* 8081. J. Biol. Chem., *177:* 533.

WEST, J. W., C. W. CARRICK, S. M. HAUGE AND E. T. MERTZ 1952 The relationship of choline and cystine to the methionine requirement of young chickens. Poultry Sci., in press.

WOMACK, M., AND W. C. ROSE 1941 The partial replacement of dietary methionine by cystine for purposes of growth. J. Biol. Chem., *141:* 375.

———— 1946 The partial replacement of dietary phenylalanine by tyrosine for purposes of growth. Ibid., *166:* 429.

DENTAL CARIES IN THE RAT, MUS NORVEGICUS

I. THE INCIDENCE AND EXTENT OF TOOTH DECAY IN THREE STRAINS OF RATS FED FINELY POWDERED, SYNTHETIC RATIONS [1,2]

JULIA O. HOLMES

Home Economics Research and Experiment Station Chemistry, University of Massachusetts, Amherst

(Received for publication August 31, 1951)

A coarse, hard-particle consistency of the diet, with resulting marked tooth-fracturing potentiality, was believed for many years to be the sine qua non in the production of tooth decay in the Norway rat (Bibby and Sedwick, '33; Rosebury, Karshan and Foley, '33; Hodge and Sognnaes, '46). Important factors leading to this "hard-particle" theory of dental caries origin in the Norway rat were: (a) the inability of the early workers (Jackson, '30; Lilly, '32; Lilly and Grace, '32–'33) to induce tooth decay in this species by feeding powdered, synthetic rations; (b) the production of rampant tooth decay by diets containing coarse, hard particles (Hoppert, Webber and Canniff, '31); and (c) the failure of Sognnaes ('41) and of Ginn ('41) to produce cavities in the teeth of rats fed hard-particle rations if the teeth of the opposing jaw were extracted, thereby preventing mastication and consequent fracturing of the cusps and injury to the enamel. However, following the successful induction of tooth decay in the cotton rat by Shaw, Schweigert, McIntire, Elvehjem and Phillips ('44) by feeding synthetic rations rich in sugar and devoid of hard particles, Sognnaes ('47, '48) demonstrated

[1] Contribution 814 of the Massachusetts Agricultural Experiment Station.

[2] These studies were aided by a contract between the Office of Naval Research, Department of the Navy and the University of Massachusetts (NR 181–485) and by a grant from the National Dairy Council.

that if a similar diet were fed to his caries-resistant Long-Evans strain of Norway rat during pregnancy and lactation, the offspring developed tooth decay when they were continued on the same ration after weaning; but if the mothers were reared on a natural-food ration, their young remained caries-resistant. Likewise, on applying the Sognnaes prenatal, preweaning cariogenic feeding technique, Shaw ('50) rendered his caries-resistant Sprague-Dawley rats susceptible to tooth decay. McClure, who at one time had produced cavities in his strain of rats by feeding them a powdered, synthetic, sugar-rich diet but who three years later reported that his colony was resistant to tooth decay ('45), succeeded in producing cavities in that colony by instituting a synthetic, cariogenic diet when the young were born ('51); two other strains of Norway rats subjected to this regimen developed cavities, but a third strain did not. Reference has been found to only one strain of Norway rat that develops marked caries on synthetic rations devoid of hard particles without being subjected to preliminary prenatal or preweaning cariogenic feeding treatment (Schlack, Taylor, Gerende, Berzinskas and Mullins, '49).

In this paper evidence is presented of the production of dental caries in three strains of Norway rats reared to weaning age by mothers on stock rations of natural foods and thereafter maintained on powdered, synthetic, sugar-rich rations devoid of hard particles. The three strains differed in their susceptibility to tooth decay. None of the strains was bred for high caries-susceptibility.

EXPERIMENTAL

Three strains of rats, two white and one black, were employed. Strain *MW* was purchased from The Wistar Institute in 1934 and inbred until 1945, when several Sprague-Dawley males were introduced into the colony. Strain *MB* was a heterozygous black rat.[3] Strain *C* was purchased in

[3] Purchased in 1943 from Carworth Farms, New City, New York.

1947 from the Connecticut Agricultural Experiment Station and, according to Hubbell ('49), was a mixture of stock of unknown origin and a few Wistar Institute males introduced in 1912 and again in 1918, and inbred since that time.

The young rats were placed on the experimental rations at 21 to 30 days of age and kept in individual wire-mesh cages on raised, wide-mesh screen floors. Distilled water was available at all times, and the rations were fed ad libitum. The length of the experimental period was 98 days for strains *MW* and *MB* rats and 98, 77, and 60 days for strain *C* rats. The rats that were fed for the 98- or 77-day periods, with few exceptions, served as controls in dental caries studies; hence almost as many litters are represented as there are rats. In contrast, those fed for a 60-day period came primarily from 15 litters. Only 6 of the rats, strain *C*, fed for 98 days, had litter mates fed for 77 days.

The basic breeding ration, I, fed during the first three years of the study ('45 to '48), contained yellow cornmeal 42, wheat middlings 25, nonfat milk solids 15, meat scraps 8, alfalfa meal 5, wheat germ 1.5, $CaCO_3$ 1.5, and NaCl 0.5%. Supplements of carrots and lettuce or cabbage were fed twice weekly, and during the interval between March and October, 1948, raw beef muscle was fed during lactation. Beginning in October, 1948, ration II was fed; it consisted of ground whole wheat 59.3, whole milk solids 29.6, meat scraps 9.9; and NaCl 1.2%. Tapwater was supplied for drinking.

The experimental rations were composed of "vitamin-free" casein [4] 15 to 25%, Phillips and Hart ('35) salt mixture 4, corn oil 4, liver fraction L [5] 4, and sucrose or glucose 63 to 73%. Prior to August, 1947, the vitamins were fed in liquid form as daily supplements apart from the ration; since then, they have been mixed into the ration in the following quantities per kilogram of ration: thiamine 4 mg, pyridoxine 4 mg, riboflavin 6 mg, 2-methyl-1,4-naphthoquinone 6 mg, calcium pantothenate 25 mg, nicotinic acid 50 mg, *p*-aminoben-

[4] Labco.
[5] Wilson.

zoic acid 300 mg, choline 1 gm, inositol 1 gm, alpha-tocopherol 50 mg, beta-carotene 10 mg, and irradiated ergosterol, 3,000 U.S.P. units.

For examination of the teeth for caries, a binocular microscope at a magnification of 30 × was used. Inspection of the enamel and dentin in the fissures was facilitated by alternately grinding and examining the molars at successive planes. Each mandible and maxilla was ground separately.

The criterion for designating the enamel as carious was the presence of soft, spongy or sticky, yellow tissue, or of soft opaque areas. Dentin was recorded as carious if the tissue was yellow and soft. In scoring for incidence of decay, a numerical value of 1 was assigned to each site of decay, a maximum of 1 being recorded for a given fissure even though both walls were carious. In scoring for extent of decay, the 1-2-3 system adopted by Schlack, Howell and Taylor ('47) was used; a value of 1 was given if only enamel was involved; 2, if the dentin was carious; and 3, if the area, irrespective of size, had undergone cavitation. Each fissure wall of the major cusps was treated as a separate area; hence, a maximum score of 6 for extent of decay was possible in a given fissure if both walls were involved in cavitation. In the narrow fissure formed by the small, fused, mesial or lateral cusps, the two walls were considered as one area of decay, and a maximum score of 3 was possible for extent of decay. In scoring the opaque areas, a value of 1 was assigned to an opaque site on any one of the surfaces of a given cusp.

RESULTS

The data are presented in table 1 as means for the various groups, together with the standard deviation from the means. Differences in the incidence and extent of tooth decay owing to differences in the quality of the maternal diet, in the amount of casein, or in the type of carbohydrate in the experimental rations were slight; hence, the data for all the rats of a given strain observed for a given period of time were averaged.

TABLE 1

Data pertaining to three strains of Norway rats reared to weaning age by mothers on natural food rations and then fed powdered, synthetic, sugar-rich rations

STRAIN OF RAT	MW	MB	C	C	C
No. of rats	106	63	29	91	112
Length of experimental period (days)	98	98	98	77	60
Mean weight gain/day, ♀	2.3 ± 0.3 (66)[1, 2]	2.6 ± 0.2 (32)	2.6 ± 0.2 (14)	2.8 ± 0.4 (54)	2.7 ± 0.4 (63)
in gm, first 60 days ♂	3.2 ± 0.6 (40)	3.7 ± 0.6 (31)	4.1 ± 0.6 (15)	4.0 ± 1.0 (37)	4.0 ± 0.3 (49)
No. of rats with caries (%)	94	98	100	99	100
Carious molars					
No./rat	5.6 ± 2.8	7.5 ± 3.7	8.0 ± 1.8	9.5 ± 1.4	9.4 ± 2.3
% of total molars	47	62	67	79	78
Cavities					
No./rat	1.0 ± 1.5	1.1 ± 1.0	4.0 ± 3.6	2.7 ± 2.8	2.1 ± 1.9
No. animals with cavities (%)	41	46	76	75	66
Incidence of decay, sites/rat	8.7 ± 5.8	14.1 ± 8.1	13.9 ± 6.6	17.1 ± 5.2	17.3 ± 5.6
Extent of decay, score/rat	14.7 ± 13.3	26.6 ± 20.1	37.8 ± 23.8	39.4 ± 24.8	42.1 ± 18.6

[1] Standard deviation.

[2] Number of rats in group.

Among the rats fed the cariogenic rations for 98 days, the degree of tooth decay was lowest in strain *MW* rats and highest in strain *C* rats. Carious lesions were detected in 94% of the 106 strain *MW* rats, in 98% of the 63 strain *MB* rats, and in 100% of the 29 strain *C* rats, the mean number of carious molars/rat being 5.6 ± 2.8, 7.5 ± 3.7, and 8.0 ± 1.8, respectively. The percentages of rats having cavities were 41, 46, and 76, respectively, and the mean number of cavities/rat was 1.0 ± 1.5, 1.1 ± 1.0, and 4.0 ± 3.6. The incidence of decay/rat was 8.7 ± 5.8, 14.1 ± 8.1, and 13.9 ± 6.6, respectively, whereas the scores for extent of decay/rat were 14.7 ± 13.3, 26.6 ± 20.1, and 37.8 ± 23.8.

In a subsequent study to determine the influence of the length of the experimental period on the caries scores in strain *C* rats, the number of rats developing carious lesions, and the incidence and extent of decay, were found to be as great in the rats observed for only 60 days as in those observed for 77 or 98 days. The number of rats having cavities and the number of cavities/rat, however, appeared to increase with increases in the length of the experimental period; the mean number of cavities in rats fed for 60, 77, and 98 days was 2.1, 2.7, and 4.0, respectively. These mean values, when plotted against length of experimental period, tended to fall on a straight line; the differences between the group means, however, were not statistically significant. For the 6 rats fed for 98 days and for their litter mates fed for 77 days, the respective mean values for carious molars were 8.0 ± 0.3 and 7.9 ± 1.7; for incidence of decay, 10.8 ± 2.1 and 12.3 ± 4.2; for extent of decay, 16.6 ± 5.8 and 15.2 ± 7.7; and for number of cavities 1.0 ± 1.3 and 0.5 ± 0.2/rat.

Widespread fracturing of the cusps, characteristic of hard-particle rations, was not found in this study. A small amount of fracturing was associated with many of the cavities, as was evidenced by the sharp angles of the cavity-contours; but when fracturing was observed, it was invariably underlaid by yellow, soft dentin. In many instances, decay had progressed to the occlusal surface without a breakdown of the

cusp wall; in other instances, cavities were found deep in the sulci with overhanging, seemingly intact, cusps. Small "slivers" of enamel occasionally had been chipped from a cusp wall beginning at the occlusal surfaces, but the areas were adjacent to yellow, soft dentin or enamel, or were at sites at which initial decay was never found.

Attrition of the cusps, characterized by a uniform wearing-away of both enamel and dentin, as found in rats fed hard-particle rations, was not observed. An uneven erosion, with more dentin than enamel being lost, resulting in smooth, shiny, cup-shaped, occlusal surfaces, was found in all rats, seemingly irrespective of the length of the feeding period. Erosion on the lingual surfaces, as described in rats fed acid beverages (Restarski, Gortner and McCay, '45), was seldom seen.

Marked variation in the number and extent of the carious lesions within a given species characterized the data, as is indicated by the high values for standard deviation of the means and by the high coefficients of variation. For the *MW* and *MB* rats fed for 98 days and for the strain *C* rats fed for 98, 77, and 60 days, the mean coefficients of variation for the data for number of carious sites were 66, 57, 33, 30 and 32%, respectively; for extent of decay they were 90, 75, 63, 63, and 44%. These coefficients indicate that the strain *C* rats were the least variable of the three strains.

DISCUSSION

The three strains of rats observed in this study developed tooth decay but differed markedly in the number of carious sites and in the severity of the lesions developed. Strain differences in caries-susceptibility have been reported for the Norway rat by Hunt and co-workers ('44) and by McClure ('51), and for the cotton rat by Schweigert and co-workers ('45). In view of these findings, it is probable that the failures of the early workers (Jackson, '30; Lilly, '32; Lilly and Grace, '32–'33) to induce tooth decay in Norway rats by feed-

ing them hard-particle-free, sugar-rich rations may have been due, in part, to the use of strains that were inherently resistant to tooth decay.

The tooth decay induced in the three strains of Norway rats, reared to weaning age by mothers on natural food diets, appears, in general, to be of the type found in cotton rats reared by mothers on natural food diets (Shaw and co-workers, '44) and found also in the caries-resistant Long-Evans strain of Norway rat made caries-susceptible by instituting a powdered, synthetic diet at the time of conception of the experimental animal (Sognnaes, '47, '48, '49). The diets used in these different studies were similar in composition and consistency, and the decay was characterized by an absence of initial fracturing. In the three strains employed in this study, particularly strain *C*, the high scores for microscopic caries associated with low cavity-incidence rule out the possibility that the lesions were the result of particle hardness. The lesions in strain *C* rats developed rapidly and were as numerous at the end of a 60-day period as at the end of a 98-day period; in 6 rats fed for only 42 days (data not presented), as many microscopic lesions were found as were observed in their litter mates fed for the longer periods. Other workers using Norway rats have not reported caries-incidence in their animals at the end of a period as short as 60 days, but Kite, Shaw and Sognnaes ('50), who scored their Norway rats on the basis of microscopic caries, found it necessary to feed their rats for a 130-day period.

A comparison of the severity of the tooth decay induced in these three strains of Norway rats and in other colonies of this species fed the powdered, synthetic rations is somewhat difficult because of differences in methods of scoring the lesions. The colony of Schlack and co-workers ('49) is presumably comparable to strain *C* rats, as indicated by the mean value of 16.0 carious sites per rat in 10 rats reared by mothers on a natural food ration, then fed a powdered, synthetic ration for 98 days and scored for incidence of decay by the same method as was used in this study. Data for other equally

caries-susceptible strains reared under comparable conditions have not been found in the literature. Sognnaes ('47, '48), by feeding a powdered synthetic ration to a caries-resistant Long-Evans strain of rats during pregnancy and lactation and by continuing the young on the same ration for 90 to 130 days, observed cavities similar in number to those in strain *C* rats reared by mothers on a natural food diet. Shaw ('50), using a caries-resistant Sprague-Dawley strain rendered caries-susceptible by the Sognnaes technique, reported values from 61 rats for carious molars and carious sites, as found in the ground tooth, which averaged 6.1/rat and 8.7/rat, respectively, and Kite, Shaw and Sognnaes ('50), using the same technique, reported similar values in 13 rats of an unspecified strain. These values, in general, are lower than those found in strain *MW* rats reared by mothers on a natural food diet. In McClure's ('51) colony, in which the powdered, synthetic rations were instituted at the birth of the young, the number of cavities that developed when the ration was adequate was similar to that in strain *MW* rats; but when the ration was inadequate in protein, salts and vitamins, the number of cavities was similar to that in strain *C* rats. McClure also found that a Sherman strain was even less susceptible to caries than his own strain but that a mixed group, composed of rats from Hunt's colony at Michigan State College and from Schlack's colony, reared on powdered, synthetic rations from birth, was exceptionally caries-susceptible; of 51 rats, 80% had cavities, and the mean number of cavities, based on 51 rats rather than on the 41 affected rats, was 10/rat. Although the mean number of cavities in this mixed group was far greater than the mean number that would be expected in strain *C* rats observed for a comparable period, the number of rats that developed cavities was not appreciably greater than the number of strain *C* rats that developed cavities; i.e., 76%.

SUMMARY

Three strains of Norway rats, two white and one black, totalling 401 rats, were employed in this study; they were

raised by mothers on natural food rations, and no strain was bred for high caries-susceptibility.

The synthetic and finely powdered rations used contained all the nutrients seemingly necessary for good growth and maintenance of health. Sucrose or glucose comprised 63 to 73% of the rations. The rations were fed for periods of 60 to 98 days.

The presence of tooth decay was determined by alternately grinding and inspecting the molars for yellow, soft enamel or yellow, soft dentin at successive planes.

Tooth decay developed in all three strains. One white strain, strain *C*, developed 60% more decayed areas, 160% more extensive areas of decay, and 400% more cavities than did the other white strain. The black strain fell midway between the two white strains in caries-susceptibility.

A 60-day feeding period for strain *C* rats was found to be as satisfactory as a period of 77 or 98 days, and an even shorter period might be sufficient.

ACKNOWLEDGMENT

The author is pleased to acknowledge the technical assistance of Mr. Leonard R. Parkinson during a part of this study.

LITERATURE CITED

BIBBY, B. G., AND H. J. SEDWICK 1933 Formation of cavities in the molar teeth of rats. J. Dent. Res., *13:* 429.

GINN, J. T. 1941 Importance of mechanical factors upon the progress of experimental dental caries in the rat. Ibid., *21:* 333.

HODGE, H. C., AND R. F. SOGNNAES 1946 Experimental caries and a discussion of mechanisms of caries inhibition by fluorine. Dental caries and fluorine. Am. Assn. for Advancement of Science, Washington, D. C., pp. 53–73.

HOPPERT, C. A., P. A. WEBBER AND T. L. CANNIFF 1931 The production of dental caries in rats fed an adequate diet. Science, *74:* 77.

HUBBELL, R. B. 1949 Personal communication.

HUNT, H. R., C. A. HOPPERT AND W. C. ERWIN 1944 Inheritance of susceptibility to caries in albino rats (Mus norvegicus). J. Dent. Res., *20:* 385.

JACKSON, C. M. 1930 The effects of high sugar diets on the growth and structure of the rat. J. Nutrition, *3:* 61.

KITE, O. W., J. H. SHAW AND R. F. SOGNNAES 1950 The prevention of experimental tooth decay by tube feeding. Ibid., *42:* 89.

LILLY, C. A. 1932 Failures to produce experimental dental caries in the white rat with a high carbohydrate diet and bacillus acidophilus or with vitamin D deficiency. Ibid., *5:* 175.

LILLY, C. A., AND J. D. GRACE 1932–33 Failure to produce dental caries with high carbohydrate and with extremely low fat diets. Proc. Soc. Exp. Biol. Med., *30:* 176.

MCCLURE, F. J. 1945 Observations on induced caries in rats. V. Effects of excessive sugar in the diet. J. Dent. Res., *24:* 239.

———— 1951 Experimental dental caries in rats. J. Nutrition, *43:* 303.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

RESTARSKI, J. S., R. A. GORTNER, JR. AND C. M. MCCAY 1945 A method of measuring the effects of acid beverages on the teeth of small laboratory animals. Science, *102:* 404.

ROSEBURY, T., M. KARSHAN AND G. FOLEY 1933 Studies, in the rat, of susceptibility to dental caries. III. The experimental production of typical dental fissure-caries and other lesions in rats, and preliminary studies of their etiology. J. Dent. Res., *13:* 379.

SCHLACK, C. A., S. R. HOWELL AND B. L. TAYLOR 1947 A procedure for preparing and examining rats' teeth for dental caries. Naval Medical Research Institute, National Naval Medical Center, Report No. 5, July 17.

SCHLACK, C. A., B. L. TAYLOR, L. J. GERENDE, V. J. BERZINSKAS AND C. E. MULLINS 1949 The effects of standard diets and some concomitant variables on the incidence of dental caries in white rats. Oral Surg., Oral Med., Oral Path., *2:* 1208.

SCHWEIGERT, B. S., J. H. SHAW, C. A. ELVEHJEM AND P. H. PHILLIPS 1945 Dental caries in the cotton rat. V. Influence of strain variation on the caries susceptibility. Proc. Soc. Exp. Biol. Med., *59:* 44.

SHAW, J. H. 1950 Effects of dietary composition on tooth decay in the albino rat. J. Nutrition, *41:* 13.

SHAW, J. H., B. S. SCHWEIGERT, J. M. MCINTIRE, C. A. ELVEHJEM AND P. H. PHILLIPS 1944 Dental caries in the cotton rat. I. Methods of study and preliminary nutritional experiments. Ibid., *28:* 333.

SOGNNAES, R. B. 1941 Studies on masticatory efficiency. IV. Mastication and experimental rat caries. Am. J. Orthodontics Oral Surg., *27:* 552.

———— 1947 A possible role of food purification in the etiology of dental caries. Science, *106:* 447.

———— 1948 Experimental rat caries. I. Production of rat caries in the presence of all known nutritional essentials and in the absence of coarse food particles and the impact of mastication. J. Nutrition, *36:* 1.

———— 1949 Experimental rat caries. II. Location, sequence and extent of carious lesions produced in the Norway rat when raised on a generally adequate, finely powdered, purified ration. Ibid., *39:* 139.

METABOLISM OF NICOTINIC ACID IN PREGNANCY[1, 2]

MARY E. LOJKIN, ANNE W. WERTZ AND CHARLOTTE G. DIETZ
Nutrition Research, Department of Home Economics, Massachusetts Agricultural Experiment Station, University of Massachusetts, Amherst

(Received for publication September 29, 1951)

In the course of an investigation of the nutritional status of pregnant women, undertaken as part of a cooperative problem in the Northeast Region (Cooperative Nutritional Status Studies, '51), some experiments on the metabolism of nicotinic acid were performed. Preliminary work indicated a marked increase in the urinary excretion of N^1-methylnicotinamide (hereafter referred to in this paper as MNA) in pregnancy, as well as a small but consistent increase in the excretion of the acid-hydrolyzable metabolites of nicotinic acid (hereafter referred to in this paper as NA).

Results of a similar nature in studies of MNA excretion have been reported by Moore et al. ('47) and later by Frazier, Porter and Humphrey ('49) and Oldham and co-workers ('49). These investigators found a definite increase in the excretion of MNA by human subjects, irrespective of intake, with the progress of pregnancy. On low niacin intakes the urinary excretion of the niacin metabolites in primigravidae often exceeded the amount of niacin ingested (Frazier et al., '49; Oldham et al., '49). Yet according to Frazier et al. ('49), the level of urinary excretion of NA was not affected by pregnancy and remained fairly constant at 5 to 8% of the intake.

[1] Contribution 818 of the Massachusetts Agricultural Experiment Station.

[2] Supported in part by R. and M. funds $9b_{1-2}$ and $9b_2$ obtained under the Research and Marketing Act of 1946. Project NE-4.

In view of these findings it was considered worthwhile to make further investigations of the metabolism of nicotinic acid in pregnancy. For this purpose, studies on human subjects were continued until the number of subjects was judged sufficient for statistical interpretation of the results. Furthermore, in order to account, if possible, for the peculiarities of the metabolism of nicotinic acid in pregnancy, it was desirable to find an experimental animal which could be used for studies under certain controlled conditions that might be unsuitable for human subjects. The albino rat was used and studies made to determine whether this animal's urinary excretion of MNA and NA in pregnancy follows the pattern observed for pregnant women.

EXPERIMENTAL

Pregnant women

Studies of the metabolism of nicotinic acid have been made on 61 women, ranging in age from 16 to 34 years. Twenty-three subjects were primiparae and the others were going through their second, third, or 4th pregnancies. Five women were used as subjects during two consecutive pregnancies. In most cases the subjects were studied during three periods; i.e., early pregnancy (4th month), late pregnancy (last month), and three months post partum. Several women were available as subjects for only two periods. Eight women were selected for more detailed studies, consisting of 5 to 8 experimental periods pre and post partum.

All the subjects who cooperated in this study were patients of a local physician.[3] They lived in their homes and ate self-selected diets. Daily food records were kept by the subjects for 7 days during each study period, and the average daily intakes of individual nutrients were calculated by a nutritionist [4] by the method described in the cooperative nutritional

[3] E. M. Holden M.D., Amherst, Mass.

[4] Gladys C. Hagan, University of Massachusetts.

status studies in the Northeast Region (Cooperative Nutritional Status Studies, '51).

In 23 women the average daily tryptophan intakes were estimated, using the data of Block and Bolling ('45), Lyman and Kuiken ('49), Dunn et al. ('49), and Neilands et al. ('49). The 23 subjects, selected at random, were considered to be representative of the total group of 61.

Daily intakes of niacin for the 24-hour period immediately preceding the sampling and for the 24-hour sampling period were used for computation of niacin recovery. The values for the urinary excretion of the metabolites of MNA and NA, expressed as a percentage of the niacin intake during the 24-hour sampling period and as a percentage of the average daily intake for the two days, were compared statistically by the method of paired samples (Snedecor, '46). There was no significant difference between the results ("t" values $\leqq 0.20$) obtained. Accordingly, the practice of reporting niacin recovery as a per cent of niacin intake only during the 24-hour sampling period was adopted in these experiments.

During the 4th day of each experimental period, 24-hour urine specimens were collected. All the samples were analyzed for MNA (Huff and Perlzweig, '47) and for total nicotinic acid (Association of Vitamin Chemists, Inc., '47). In several cases the excretion of N^1-methylnicotinamide-6-pyridone-3-carboxylamide [5] was also determined (Rosen et al., '49).

Results and discussion

Twenty-four-hour excretions of MNA and NA, the percentage recoveries of these metabolites in the urine of pregnant (pre and post partum) women, and the average daily intakes of niacin and protein are presented in table 1.

The results of experiments performed individually on 61 women are reported here as averages computed separately for 23 primiparae and 38 multiparae.

[5] The authors are indebted to Dr. J. W. Huff for supplying the 6-pyridone used in these studies.

TABLE 1

Urinary excretion of N¹-methylnicotinamide and total nicotinic acid by women in early pregnancy (E), late pregnancy (L), and three months post partum (P)

PERIOD OF PREGNANCY	EXCRETION				INTAKE		
	N¹-methylnicotinamide		Total nicotinic acid		Niacin		Protein
					From 7-day food records	On sampling days	From 7-day food records
	mg per 24 hr.	*% in niacin intake*[1]	*mg per 24 hr.*	*% in niacin intake*	*mg per 24 hr.*	*mg per 24 hr.*	*gm per 24 hr.*
Average results for 23 primiparae							
E	7.6 ± 0.60[2]	66[3] ± 8.7	1.4 ± 0.15	14 ± 2.0	12.3 ± 0.90	12.2 ± 1.18	66 ± 4
L	11.7 ± 0.90	88 ± 8.8	1.5 ± 0.15	15 ± 2.3	13.6 ± 0.71	12.8 ± 1.11	71 ± 3
P	4.2 ± 0.37	40 ± 3.4	1.0 ± 0.05	7 ± 0.8	13.1 ± 0.72	14.8 ± 1.24	64 ± 3
"t" values for differences among the 3 study periods							
E vs L	3.84[4]	2.18[5]	1.46	0.64	0.60	0.54	0.18
E vs P	6.51[4]	5.46[4]	3.70[4]	3.50[4]	0.40	1.55	0.75
L vs P	10.42[4]	6.89[4]	4.50[4]	3.72[4]	0.07	1.31	1.79
Average results for 38 multiparae							
E	8.7 ± 0.53	72 ± 8.5	1.6 ± 0.15	15 ± 2.1	13.0 ± 0.66	13.1 ± 1.21	67 ± 2
L	15.6 ± 0.89	120 ± 9.7	2.2 ± 0.18	19 ± 2.1	12.9 ± 0.58	13.0 ± 0.63	67 ± 2
P	5.0 ± 0.31	39 ± 4.2	1.2 ± 0.12	11 ± 1.3	12.8 ± 0.60	13.4 ± 1.24	63 ± 3
"t" values for differences among the 3 study periods							
E vs L	5.73[4]	3.25[4]	2.60[5]	1.06	0.23	0.06	0.19
E vs P	5.79[4]	3.30[4]	2.90[4]	2.77[5]	0.05	0.55	0.10
L vs P	11.55[4]	7.63[4]	3.68[4]	2.90[4]	0.78	0.33	1.20
"t" values for differences between primiparae and multiparae							
E	0.47	0.49	0.20	1.57	0.63	0.52	0.32
L	2.06[5]	2.21[5]	2.08[5]	1.33	0.73	0.17	0.86
P	1.57	0.10	1.47	1.81	0.30	0.77	0.18

[1] N¹-methylnicotinamide computed as nicotinic acid equivalent.

[2] Standard error of the mean.

[3] Average of the individual percentages of intake.

[4] Significant at $p \leq 1\%$.

[5] Significant at $p \leq 5\%$.

Excretion of MNA. The 24-hour excretions of MNA by the individual subjects in the two groups varied within the following ranges, respectively: 4.3 to 12.0 and 4.2 to 13.0 mg in early pregnancy; 6.6 to 25.7 and 8.9 to 32.9 mg in late pregnancy; and 1.7 to 6.7 and 2.6 to 8.7 mg three months post partum.

Wide variations in the daily urinary excretion of MNA have been found by other investigators. Values have been reported ranging from 3.0 to 17 mg (Huff and Perlzweig, '47) and 3.5 to 9.1 mg per day (Hochberg et al., '45) for 67 and 8 normal men, respectively, and from 4.3 to 8.8 mg (Perlzweig et al., '47) and 3.3 to 9.7 mg per day (Frazier et al., '49) for three and 12 normal women, respectively. For 5 primigravidae the ranges were (Frazier et al., '49) 7.7 to 12.2 mg of MNA per day during the 5th and 12.0 to 20.0 mg per day during the 9th month of pregnancy.

The results for each group of subjects in table 1 indicate higher levels of MNA excretion by women during pregnancy than in the nonpregnant state. Moreover there is an augmentation in the 24-hour urinary excretion of MNA with the duration of pregnancy. Similar trends in the excretion of MNA were also observed when the results were reported as a percentage of the niacin ingested during the 24-hour collection period. The increases were statistically significant, as may be seen from the "t" values obtained by the method of paired comparison. Correlation coefficients between MNA excretion and niacin intake, computed separately for the subjects in early pregnancy, late pregnancy, and post partum (0.11, 0.03 and 0.08, respectively) were not significant, indicating that under the conditions of the experiment the MNA excretion was independent of niacin intake. These results were in agreement with the previously mentioned reports of other investigators.

Although on the average (table 1) the excretion of MNA rose with the duration of pregnancy, a more detailed study of individual subjects during several periods of advanced pregnancy and post partum indicated that in some subjects there

was no steady gradual augmentation from month to month, with maximum excretion in the 9th month of pregnancy. Rather, there were uneven changes, with highest excretions occasionally occurring during the 8th month, or even post partum. The excretion of MNA did not diminish to the nonpregnancy levels immediately post partum. Levels one week post partum varied widely in the different individuals: in about 50% of the women studied there was a marked decrease and in about 10% a considerable increase, whereas in the other cases there was little change in the excretion of MNA, with levels remaining in the neighborhood of the pre partum values. The excretions were approaching the nonpregnancy levels about one month post partum.

In late pregnancy the excretion of MNA, calculated as a per cent of niacin intake during the 24-hour sampling period, varied within the limits of 50 to 270%, with an average of 107% for the 61 subjects studied. This excess of excretion over intake suggested two different explanations: either that other sources of niacin were available to the body, or that erroneously high results were obtained because of the possible presence in the pregnant women's urine of interfering substances, which fluoresce under the conditions of the acetone method as used for MNA analysis.

In order to investigate the latter possibility, samples of urine from 10 subjects were analyzed for MNA by both the acetone (Huff and Perlzweig, '47) and the butanol method (Huff and Perlzweig, '43a, b). The agreement between these two methods of analysis for MNA has been reported (Huff and Perlzweig, '47) to be within 10% for normal subjects. The results obtained in these experiments were found to agree within 8%. In view of the good agreement and the reported specificity of the acetone method, it seems reasonable to conclude that the fluorescence-producing substance is actually MNA. Another point in favor of the view that the substance measured was actually MNA is the report by Kaser et al. ('47) to the effect that in pregnancy the increases in the excretion of MNA following administration of a test dose of

nicotinamide are higher than those observed in nonpregnant subjects.

Excretion of N^1-methylnicotinamide-6-pyridone-3-carboxylamide. Determinations of the excretion of the 6-pyridone were performed primarily to investigate whether the high levels of MNA excretion in pregnancy might be due to an increased storage of this metabolite in the tissues because of some impairment in the oxidizing processes, which convert MNA into its 6-pyridone. In 10 studies of the urinary excretion of the 6-pyridone it was found that the subjects whose daily excretions of MNA were 7.1 ± 1.9 mg in early and 17.9 ± 2.60 mg in late pregnancy excreted 10.0 ± 2.9 mg and 28.5 ± 4.10 mg of the 6-pyridone, respectively. It is evident that the increases in excretion of MNA in pregnancy were not compensated for by corresponding decreases in the excretion of the 6-pyridone. Rather, there was a tendency for higher levels of 6-pyridone excretion with increased levels of MNA excretion. The ratios of the 6-pyridone to the MNA urinary content varied from 1.1 to 2.1 and averaged 1.6 ± 0.12. These results were in agreement with the findings of other investigators. Normal human urine has been reported to contain the 6-pyridone in amounts either equal to those of MNA (Perlzweig et al., '49) or exceeding them by factors of two to 4 (Holman and de Lange, '50). In view of the limited accuracy (ranging to 30%) of the method of analysis for the 6-pyridone (Rosen et al., '49) and some inconsistency in the ratio of the 6-pyridone to the MNA obtained in these experiments, the number of determinations of the 6-pyridone is not considered sufficient for any definite conclusions concerning the effect of pregnancy on the 6-pyridone excretion. It can be seen, however, from the results obtained, that the amounts of the 6-pyridone excreted by women during the advanced state of pregnancy were higher than the values of 5.5 to 9.5 mg (Rosen et al., '49) and 14.2 mg (Holman and de Lange, '50) per 24 hours which have been reported for normal subjects.

Excretion of total nicotinic acid. A wide variation was found in the excretion of NA by the individual subjects, with values ranging within the following limits for the individual primiparae and multiparae, respectively: 0.8 to 2.4 mg and 0.4 to 3.4 mg in early pregnancy, 0.8 to 3.0 mg and 0.7 to 4.7 mg in late pregnancy, and 0.3 to 1.4 mg and 0.5 to 3.8 mg three months post partum. Reports in the literature of excretions ranging from 0.7 to 2.2 mg, with an average of 1.2 mg, for 33 normal men (Sarett et al., '42), and from 0.6 to 2.0 mg for 45 determinations on young women, with averages in the neighborhood of 1.0 mg (Oldham et al., '46), are in good agreement with the results of this study. The reported values, ranging from 0.9 to 1.7 with an average of 1.3 mg, for the daily NA excretion of 5 women in the 9th month of pregnancy (Frazier et al., '49) are somewhat lower than the corresponding values observed in this investigation.

Comparison of the daily urinary excretion as well as of the percentage recovery of NA during the three periods of study (table 1) indicates higher levels of excretion of this metabolite in pregnancy. The "t" values indicate that the differences between the excretion of NA in pregnancy and post partum were significant, whereas the rises in the excretion of NA with the progress of pregnancy were of little or no significance (5% and 20% probability levels for multiparae and primiparae, respectively).

Correlation coefficients between total nicotinic acid excretion and the niacin intake during the sampling period, calculated separately for the subjects in early pregnancy, late pregnancy and post partum (0.03, 0.07, and 0.01, respectively) indicate that under the conditions of the experiment the urinary excretion of NA is not affected by niacin intake.

From table 1 it may be seen that although the changes in the excretion of MNA resulting from pregnancy were considerably larger than the corresponding changes in the excretion of NA, the trends of the changes were similar. A correlation coefficient of 0.594 for 173 degrees of freedom established the existence of a significant correlation between

the urinary contents of MNA and NA, when the excretion of these metabolites is influenced by pregnancy.

Excretion of metabolites of nicotinic acid by primiparae and multiparae. Comparison of the results obtained for the multiparae and the primiparae (table 1) indicates higher average excretions of both MNA and NA by the multiparae. The differences were significant at the 5% probability level for excretions during the period of late pregnancy only. Most of the multiparae were older and heavier than the primiparae, the average ages for the two groups being 26.6 ± 0.5 [a] and 22.5 ± 0.7 years, and the average weights 137 ± 4 and 120 ± 3 pounds, respectively. Statistical examination of the data revealed that the differing excretion of the metabolites of nicotinic acid could not be attributed to differences in the weight, age, and food consumption of the two groups of subjects. Evidently some other factors are responsible for the somewhat higher levels of excretion of MNA and NA by the multiparae.

Niacin, tryptophan, and total protein intake. The finding that the sum of the MNA and NA excreted by women in the state of late pregnancy averaged 125% (table 1) of the niacin ingested during the sampling period suggested that other sources of niacin were available to the body. The observed high levels of excretion of the 6-pyridone by most of the women who were studied for 6-pyridone excretions gave additional weight to this view. In order to investigate further the relationship between the sources of niacin and the excretion of its metabolites in pregnancy, the intakes of both niacin and its precursor, tryptophan, as well as the quantity and quality of the protein consumed by the subjects, were estimated from the food records.

It can be seen from the data presented in table 1 that there was good agreement between the average values for daily niacin intakes estimated from the 7-day food records and those obtained from the sampling day food records. The

[a] Standard error of the mean.

"t" values indicate the absence of any significant differences in the consumption of niacin and of protein among the women in the three states of pregnancy, and also between the intakes of these nutrients by the primiparae and the multiparae.

The tryptophan consumption computed from 64 weekly food records obtained from 23 subjects was found to be within the limits of 0.37 to 1.53 gm per day, with averages of 0.88 ± 0.04, 0.90 ± 0.05, and 0.87 ± 0.06 gm, respectively, for the three periods of the study. The differences between these values for tryptophan intake were not significant ("t" values ≤ 0.96). Examination of the food records indicated that the protein consumed by the individual subjects, as well as by the same women during the different states of pregnancy and post partum, did not vary much in its tryptophan content. This observation was confirmed by the value of 0.99 for the correlation coefficient between protein and tryptophan intakes, calculated from 64 food records. Approximately 34% of the protein was furnished by milk, 27% by meat products, and 6% by eggs. There was a negligible consumption (approximately 0.7%) of tryptophan-deficient proteins, such as gelatin and zein, which, by upsetting the amino acid equilibrium, may impair the metabolic processes involved in the tryptophan-niacin interrelationship. It is evident from these results that, under the conditions of these experiments, the amount of tryptophan and the amount and character of the protein consumed by the women had no apparent effect on changes in the levels of excretion of the metabolites of nicotinic acid in pregnancy.

Examination of the individual data obtained from 10 subjects on their daily intake of tryptophan (0.69 to 1.53 gm) and of niacin (9.7 to 18.9 mg) and data on their excretions of 6-pyridone, MNA, and NA revealed that the total daily excretion of these metabolites by some of the pregnant women exceeded the sum of the nicotinic acid consumed and of the nicotinic acid that could be synthesized from the tryptophan ingested. These results suggest the conclusion that the maternal organism has to draw from the nicotinic acid com-

pounds stored in the body in order to compensate for the high excretion of the metabolic products of nicotinic acid.

EXPERIMENTAL

Rats

The albino rats in these experiments were from a strain originally obtained from the Connecticut Agricultural Experiment Station. The animals were fed a stock breeder's diet consisting of 10% meat scraps, 60% whole wheat flour, and 30% whole milk powder, to which 1.5% of sodium chloride was added. The diet contained 44 μg of niacin per gram, according to analysis by the microbiological method (Association of Vitamin Chemists, Inc., '47). Estimation on the basis of the data reported by Block and Bolling ('45) and by Lyman and Kuiken ('49) gave a value of approximately 2.4 mg of tryptophan per gram of diet.

Twenty-four cases of the pregnancy of rats were studied. In 18 cases the animals were fed ad libitum. It was observed that the food consumption of the rats usually increased with the duration of pregnancy and that the coincident increase in niacin and tryptophan intakes might mask the effect of pregnancy. In 6 experiments the intake of niacin and tryptophan was kept constant throughout the period of gestation; this was done by restricting the amount of stock ration to the levels which each individual rat consumed during the pre-pregnancy or early pregnancy period. The requirement for a higher caloric intake as pregnancy progressed was met by adding dextrose to the diet.

Twenty-four-hour fasting urine collections were made at intervals before, during, and after the 21 days' gestation period, for determination of MNA and NA.

Results and discussion

The daily urinary excretions of MNA and NA, the percentage recovery of the niacin ingested during the 24 hours preceding the sampling period, the weights of the animals,

and their average niacin and tryptophan intakes are listed in table 2 for two groups of rats.

The results are reported for 6 study periods, as follows: before birth (within 7 days before conception); early pregnancy (up to the 8th day); advanced pregnancy (up to the 18th day); during the week of birth (three days before birth and three days after parturition); 4 to 10 days post partum; and 10 to 20 days post partum.

Excretion of metabolic products of nicotinic acid. Considable individual variation was found in the daily excretion of the metabolites of nicotinic acid by the different rats, as can be seen from the standard errors of the means in table 2. That individual rats vary widely in their excretion of MNA has also been observed by Perlzweig et al. ('43), who reported MNA excretions of 355 μg and 1,030 μg per 24 hours for rats of the Vanderbilt strain.

The average daily excretions by the pregnant rats as well as the percentage recovery of both metabolites of nicotinic acid increased from the time of conception and continued to rise steadily with the duration of pregnancy. The differences in the results obtained for the three periods of pregnancy were significant for both MNA and NA, as is indicated by the "t" values (table 2). The excretions usually remained above the normal level for one or two weeks after birth. There was a significant correlation between the amounts of MNA and NA excreted by the rats during pregnancy and post partum.

A comparison was made of the results obtained from the group of rats fed ad libitum and those from the group receiving a restricted food intake. In spite of the fact that the ad libitum group was consuming increasing amounts of niacin and tryptophan with the duration of pregnancy, and that the other group had a constant intake of these nutrients throughout pregnancy, the increases in the excretion of the metabolites of nicotinic acid were approximately equal in the two groups (table 2). These findings suggest that pregnancy, and not the intakes of niacin and tryptophan, was the

TABLE 2

Urinary excretion of N¹-methylnicotinamide and total nicotinic acid by pregnant rats

PERIOD OF PREGNANCY	EXCRETION				INTAKE		WEIGHT OF RATS
	N¹-methylnicotinamide		Total nicotinic acid		Niacin	Tryptophan	
	μg per 24 hr.	*% of niacin intake* [1]	*μg per 24 hr.*	*% of niacin intake*	*μg per 24 hr.*	*mg per 24 hr.*	*gm*
Unrestricted food intake average for 18 pregnancies							
Before	291 ± 20 [2]	32[3] ± 1.7	78 ± 4.8	10 ± 0.57	763 ± 30	48 ± 1.9	318 ± 5.4
Early	338 ± 22	37 ± 1.5	90 ± 1.9	11 ± 0.06	797 ± 35	50 ± 2.2	332 ± 6.5
Advanced	412 ± 22	43 ± 1.8	109 ± 1.6	13 ± 0.06	872 ± 24	55 ± 1.5	353 ± 5.4
Week of birth	552 ± 43	55 ± 2.8	122 ± 6.8	14 ± 0.08	897 ± 30	56 ± 1.9	371 ± 6.7
4–10 days post partum	401 ± 19	47 ± 3.0	96 ± 5.4	13 ± 1.08	754 ± 22	47 ± 1.4	341 ± 6.1
10–20 days post partum	336 ± 26	38 ± 2.0	84 ± 7.0	11 ± 1.00	802 ± 31	50 ± 1.9	340 ± 8.4
Restricted food intake average for 6 pregnancies							
Before	257 ± 34	27 ± 1.3	76 ± 2.2	9 ± 0.87	804 ± 50	50 ± 3.1	357 ± 6.8
Early	293 ± 13	32 ± 1.5	79 ± 2.1	10 ± 0.06	821 ± 29	51 ± 1.8	374 ± 8.2
Advanced	329 ± 22	34 ± 1.5	87 ± 2.2	11 ± 0.07	818 ± 23	51 ± 1.4	397 ± 6.2
Week of birth	516 ± 46	53 ± 4.4	112 ± 5.9	14 ± 0.06	817 ± 25	51 ± 1.6	406 ± 12
4–10 days post partum	407 ± 61	48 ± 7.0	87 ± 14	12 ± 1.90	758 ± 64	47 ± 4.0	361 ± 25
"t" values for differences among results for 4 study periods, 24 cases of pregnancy							
Difference between							
Early and advanced	3.11 [4]	4.20 [4]	2.85 [4]	3.07 [4]			
Advanced and week of birth	3.35 [4]	5.36 [4]	4.04 [4]	3.08 [4]			
Week of birth and 4–10 days post partum	4.65 [4]	3.33 [4]	4.08 [4]	2.06			

[1] N¹-methylnicotinamide computed as nicotinic acid equivalent.
[2] Standard error of the mean.
[3] Average of the individual percentages of intake.
[4] Significant at $p \leq 1\%$.

predominant factor influencing the excretion of the metabolites of nicotinic acid by the rats, under the conditions of this experiment.

Factors influencing the niacin metabolism of the pregnant rat are under further investigation in these laboratories at the present time.

GENERAL DISCUSSION

There are marked differences in the nicotinic acid metabolism of pregnant as compared to normal subjects, which manifest themselves in definite increases in urinary output of both the methylated and the acid-hydrolyzable metabolites of nicotinic acid. The theory that high levels of MNA excretion could be due to the inability of the pregnant body to oxidize MNA into its 6-pyridone has been investigated and rejected, as there were no decreases in the urinary content of the 6-pyridone which could account for the increases in MNA. Furthermore, in the rat, the animal's normal excretion of the 6-pyridone derivative amounts to only about 6% of the MNA excretion (Perlzweig et al., '49), and the increases in the excretion of MNA in pregnancy observed in these experiments were as high as 90% of the pre-pregnancy values.

The frequently observed excretion of the metabolites of nicotinic acid by pregnant women in amounts exceeding the niacin intake probably can be attributed not only to synthesis of niacin but also to utilization of the nicotinic acid reserves of the body. Nicotinic acid, whether ingested or synthesized from tryptophan, is known to be utilized for the formation of coenzymes I and II, which are stored in the body. The increased niacin metabolism of pregnancy can be expected to result in increased utilization of the coenzymes, with corresponding rises in excretion of the metabolites of nicotinic acid. These processes would be expected to be governed by the hormones in the pregnant body. That the rates of excretion of MNA can be changed, at least temporarily, by the action of hormones has been reported by Beher and Gaebler ('50), who injected anterior pituitary growth preparations and testosterone preparations into dogs. The fact that the

excretions of MNA by both pregnant women and pregnant rats in many cases did not drop immediately after delivery, and occasionally even reached higher levels, suggests that increased metabolism of niacin in pregnancy cannot be accounted for, at least entirely, by the added metabolism of the fetus and the placenta. The factors in pregnancy that stimulate high excretion of the metabolites of nicotinic acid still remain unknown.

Studies of the niacin metabolism of pregnant rats revealed that in these animals the urinary excretion of MNA and NA in pregnancy followed the pattern observed for pregnant women. There was, however, a marked difference in the percentage recovery of the nicotinic acid between the animals and the human subjects. In advanced pregnancy the women excreted the metabolites of nicotinic acid in amounts exceeding the intake, whereas the rat's maximum excretion of the metabolites in this study amounted to about 69% of the niacin intake during the 24 hours preceding the collection period. This difference could be due to species difference, to the fact that preformed niacin is not a dietary essential for the rat, and also, to a certain extent, to the difference in the conditions of the experiments. The woman had a normal food intake during the sampling period, whereas the rat's specimens were collected during a 24-hour fasting period. Another explanation of the difference lies in the results of Perlzweig et al. ('49). These investigators reported that the urinary recovery of dietary supplements of nicotinamide amounts to approximately 90% in human beings, whereas in rats it amounts to about 70%, leaving 30% unaccounted for.

In other respects the rat's niacin metabolism in pregnancy bears a resemblance to that of the pregnant woman. In both cases pregnancy brought about increases in the urinary excretion of MNA and NA, and the increased levels of excretion of these metabolic products often persisted for some time after parturition.

The rat can be considered a suitable animal for studies of the metabolism of nicotinic acid under strictly controlled

conditions, which might assist in understanding the observed augmentation in the excretion of the metabolites of nicotinic acid in pregnancy.

SUMMARY

A study was made of the metabolism of nicotinic acid in 23 primiparae and 38 multiparae during two periods of pregnancy and one period post partum. In addition, a similar investigation was performed for several periods before, during, and after the 21 days' gestation period of 24 pregnancies of albino rats.

Pregnant women

1. There was a marked increase in the urinary excretion of MNA by human subjects in pregnancy. The levels of excretion of this metabolite rose significantly with progress of pregnancy and remained above nonpregnancy levels for some time after parturition.

2. There was a less marked but statistically significant increase in the excretion of NA in pregnancy, with almost no augmentation in the levels of its excretion with advancement of the duration of pregnancy.

3. Similar trends were also observed when the above findings were expressed as percentages of niacin intake.

4. There was a considerable variation in the amounts of MNA and of NA excreted by individual subjects, and also a marked individual variation in the increases in the levels of excretion of these metabolites resulting from pregnancy.

5. Under the conditions of these experiments there was no correlation between the excretion of MNA and NA and the intakes of niacin, tryptophan, and total protein.

6. The sum of the excretions of NA, MNA, and the 6-pyridone of MNA, which was determined for several subjects in late pregnancy, often exceeded the sum of niacin ingested and the niacin available from the tryptophan consumed.

Pregnant rats

1. In rats there was a significant increase in the excretion of both MNA and NA in pregnancy. The levels of excretion

of both metabolites increased with duration of pregnancy and returned to normal in approximately two or three weeks post partum.

2. Similar trends in the changes in levels of excretion were found when the results were expressed as percentages of niacin intake.

3. There was much individual variation in the amounts of MNA and NA excreted and in the increases in the levels of their excretion in pregnancy.

4. Under the conditions of these experiments the sum of the NMA and NA excreted by the rats in late pregnancy averaged only 69% of the niacin intake, as compared to the excretions of these metabolites by human subjects, which averaged 125% of the niacin intake.

ACKNOWLEDGMENTS

The authors are grateful to Mrs. Marion M. Berry and Miss Ellen H. Morse, who were responsible for part of the microbiological determinations, and to Miss Louise P. Guild for temporary technical assistance.

LITERATURE CITED

ASSOCIATION OF VITAMIN CHEMISTS, INC. 1947 Methods of Vitamin Assay. Interscience Publishers, Inc., New York, N. Y.

BEHER, W. T., AND O. H. GAEBLER 1950 Observations on niacin, riboflavin, allantoin, ascorbic acid, and vitamin A during anabolism induced by hormones. J. Nutrition, *41:* 447.

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Ill.

COOPERATIVE NUTRITIONAL STATUS STUDIES IN THE NORTHEAST REGION 1951 I. Techniques. Northeast Regional Publication 5. Cornell Univ. Agr. Exp. Sta., Memoir 307.

DUNN, M. S., M. N. CAMIEN, S. EIDUSON AND R. B. MALIN 1949 The nutritive value of canned foods. I. Amino acid content of fish and meat products. J. Nutrition, *39:* 177.

FRAZIER, E. I., T. PORTER AND M. J. HUMPHREY 1949 The utilization of nicotinic acid by pregnant women. Ibid., *37:* 393.

HOCHBERG, M., D. MELNICK AND B. L. OSER 1945 Chemical determination and urinary excretion of the metabolite N^1-methylnicotinamide. J. Biol. Chem., *158:* 265.

HOLMAN, W. I. M., AND D. J. DE LANGE 1950 Role of tryptophane and other amino acids in the metabolism of nicotinic acid by humans. Nature, *166:* 468.

HUFF, J. W., AND W. A. PERLZWEIG 1943a N^1-methylnicotinamide, a metabolite of nicotinic acid in the urine. J. Biol. Chem., *150:* 395.

———— 1943b A rapid method of estimating N^1-methylnicotinamide in urine. Ibid., *150:* 483.

———— 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. II. A sensitive method for the determination of N^1-methylnicotinamide in urine. Ibid., *167:* 157.

KASER, M. M., P. JONES, G. S. MCCLELLAN, R. O. CANNON AND W. J. DARBY 1947 Preliminary observations on ascorbic acid and the vitamin B-complex during pregnancy and lactation. Fed. Proc., *6:* 411.

LYMAN, C. M., AND K. A. KUIKEN 1949 The amino acid composition of meat and some other foods. Texas Agr. Sta. Bull. 708.

MOORE, M. C., M. B. PURDY, E. J. GIBBENS, M. E. HOLLINGER AND G. GOLDSMITH 1947 Food habits of women during pregnancy. J. Am. Diet. Assn., *23:* 847.

NEILANDS, J. B., R. J. SIRNY, I. SOHLJELL, F. M. STRONG AND C. A. ELVEHJEM 1949 The nutritive value of canned foods. II. Amino acid content of fish and meat products. J. Nutrition, *39:* 187.

OLDHAM, H. G., M. V. DAVIS AND L. J. ROBERTS 1946 Thiamine excretions and blood levels of young women on diets containing varying levels of the B vitamins, with some observations on niacin and pantothenic acid. Ibid., *32:* 163.

OLDHAM, H., B. B. SHEFT AND T. PORTER 1949 Biochemical studies on primigravidae on self-selected diets differing greatly in nutritive value. Fed. Proc., *8:* 391.

PERLZWEIG, W. A., M. L. C. BERNHEIM AND F. BERNHEIM 1943 The methylation of nicotinamide by rat liver *in vitro*. J. Biol. Chem., *150:* 401.

PERLZWEIG, W. A., F. ROSEN, N. LEVITAS AND J. ROBINSON 1947 The excretion of nicotinic acid derivatives after ingestion of tryptophane by man. Ibid., *167:* 511.

PERLZWEIG, W. A., F. ROSEN, I. G. LEDER, S. HUNTER AND P. B. PEARSON 1949 Comparative studies in niacin metabolism. Fed. Proc., *8:* 236.

ROSEN, F., W. A. PERLZWEIG AND I. G. LEDER 1949 A fluorometric method for the determination of the 6-pyridone of N^1-methylnicotinamide in urine. J. Biol. Chem., *179:* 157.

SARETT, H. P., J. W. HUFF AND W. A. PERLZWEIG 1942 Studies in nicotinic acid metabolism. I. The fate of nicotinic acid in man. J. Nutrition, *23:* 23.

SNEDECOR, G. W. 1946 Statistical Methods. Iowa State College Press, Ames.

THE RELATIVE UTILIZATION OF ALPHA-TOCOPHEROL AND ALPHA-TOCOPHERYL ACETATE BY HUMANS

ERLING F. WEEK, FRANK J. SEVIGNE AND MARIE E. ELLIS
Research Laboratory, Collett-Week-Nibecker, Inc.,
South San Francisco, California

(Received for publication October 1, 1951)

It has been demonstrated by Harris and Ludwig ('49) that esterified alpha-tocopherols exhibit a greater biological potency than equivalent quantities of free alpha-tocopherols when the criterion of comparison is the prevention of fetal resorption in rats (Mason and Harris, '47). The potency ratio of the esters to the free forms by this criterion was shown to be 1.62. This relationship was found to be the same, irrespective of whether natural alpha-tocopherol (d-alpha-tocopherol) was compared with its ester, or synthetic alpha-tocopherol (dl-alpha-tocopherol) with its ester.

Harris and Ludwig tentatively explained their results on the basis that tocopherol esters are relatively more resistant than free tocopherol to oxidative destruction, such as might occur prior to absorption in the gastrointestinal tracts of the assay rats.

In the case of another labile oil-soluble vitamin, vitamin A, the present authors have observed that the relative utilization of esters versus the free form by humans cannot be directly deduced from animal data (Week and Sevigne, '49a,b; '50).

The foregoing considerations suggest the need for data bearing more directly on the question of the relative efficacy of free and esterified alpha-tocopherol in humans. The development of an accurate method for estimation of total tocopherol in serum by Quaife and Harris ('44) has made possible

the study of the tocopherol content of the blood before and after supplementation with vitamin E. The application of this assay technique to a series of blood samples provides a means of determining the relative absorption of varying forms of tocopherols by constructing and comparing the resulting blood persistence curves (Quaife et al., '49).

We define the "blood persistence curve" as the curve representing the content of the substance under study per unit volume of blood or serum, plotted against time. Zero for the time coordinate is the time of ingestion of a substantial quantity of the substance under study.

Dr. Hans Popper has used the term "blood tolerance curve" to convey the same meaning in connection with vitamin A studies (Popper and Steigmann, '43), and we have followed this terminology in our own vitamin A studies. However, we have chosen to use the term "blood persistence curve" in the present paper because of the undesirable doctrinal connotations of the other term.

METHOD

Synthetic [1] dl-alpha-tocopherol ($E^{1\%}_{1\,cm}$, 292 mμ = 72.0) and dl-alpha-tocopheryl acetate ($E^{1\%}_{1\,cm}$, 285 mμ = 45.0) were used as the sources of vitamin E. Each of these respective preparations was diluted with corn oil to the equivalent concentration of 200 mg of dl-alpha-tocopherol per gram, based on the ratio of the molecular weights of alpha-tocopherol and alpha-tocopheryl acetate.

The supplements for administration were prepared by accurately weighing respective quantities of these dilutions, containing the equivalent of 1,000 mg of dl-alpha-tocopherol, into gelatin slip-joint capsules.

The subjects consisted of 10 men and 10 women in normal health and not under medical treatment or on special diets.

The supplements were fed at the breakfast meal and samples of blood were taken by venous puncture before feeding the vitamin E supplements and at two, 4, 5, 6, 8 and 24 hours

[1] Merck.

afterwards. For each subject a minimum interval of 7 days elapsed between supplements.

After eating the breakfast meal, the subjects were free to pursue their normal daily activities, reporting only at the specified times to have blood samples taken. They were instructed to eat light meals composed of low-fat foods and to eat the same foods on each respective test day.

A system of rotation in administering the respective vitamin E concentrates to the various subjects was employed in order to minimize variations in the persistence curves which could be attributed to the relative order in which the preparations were fed.

The blood samples were analyzed in duplicate by the method of Quaife and Harris ('44). Vitamin E was estimated by the Emmerie-Engel color reaction (Emmerie and Engel, '39), using an Evelyn colorimeter equipped with a 520-mμ filter and calibrated against synthetic dl-alpha-tocopherol.

RESULTS

The individual data for the 20 subjects are shown in table 1.

From the data in table 1, the total area under the individual persistence curves for the zero to 24-hour period was calculated according to a method previously described (Week and Sevigne, '50). This area is expressed in terms of microgram-hours, and can be said to represent the average increase in the vitamin E serum level from the normal (zero hour) level. These calculations are summarized in table 2.

DISCUSSION

An analysis of variance of the data in table 2 was made according to the conventional statistical method described by Snedecor ('46). The results show that the biological response to dl-alpha-tocopherol was significantly ($P < 5\%$) greater than that to dl-alpha-tocopheryl acetate. The ratio of the mean values for 20 subjects was

$$\frac{\text{dl-alpha-tocopherol (microgram-hours)}}{\text{dl-alpha-tocopheryl acetate (microgram-hours)}} = 1.35.$$

TABLE 1

Serum vitamin E levels expressed as micrograms per 100 ml for 20 subjects after ingestion of dl-alpha-tocopherol and dl-alpha-tocopheryl acetate supplements, each containing 1,000 mg of the vitamin

SUBJECT AND SEX		AGE	WEIGHT	HEIGHT	TIME IN HOURS AFTER TAKING SUPPLEMENT OF												
					dl-alpha-tocopherol							dl-alpha-tocopheryl acetate					
					0	2	4	5	6	8	24	0	2	4	5	6	8	24
		yr.	*lb.*	*in.*														
17	M	33	150	69	1238	1380	1588	1721	2263	1655	1721	1222	1522	1506	1506	1572	1656	1714
18	M	32	148	71	1277	1294	2036	3627	3702	3186	2319	1505	1547	1897	2505	3539	3080	2430
33	M	28	206	72	1509	1584	1509	1893	2084	2218	2084	1500	1434	1775	2467	2717	2242	2067
32	M	22	185	71	810	810	851	943	1460	1510	1060	1098	1181	1198	1365	1523	1498	1590
30	M	31	170	69	1162	962	1078	1195	1362	1453	1987	1122	1230	1247	1288	1730	1622	1522
14	M	33	185	71	1419	1469	2085	2685	3110	2619	1977	1612	1487	1529	1971	2337	2087	1971
26	M	35	204	70	1043	1184	1343	1643	1959	1751	1709	1406	1506	1572	2106	2164	2331	1822
31	M	25	170	73	796	721	954	971	1221	1788	1279	728	861	803	1453	1328	1170	1028
15	M	55	200	70	1481	1881	4206	5131	4631	4206	2623	1714	1797	1939	3314	3356	2547	2622
28	M	34	235	76	1535	1752	1744	2352	3844	3444	2002	1830	1946	2071	2763	2830	2721	2330
				Average	1227	1304	1739	2216	2564	2383	1876	1374	1451	1554	2074	2310	2095	1910
44	F	28	150	66	1228	1211	1428	2086	2086	2086	2045	1250	1375	1442	1933	2400	1800	1783
45	F	44	153	65	1908	2083	3317	4783	5150	3767	3317	1785	1802	2318	2860	2602	2802	2268
46	F	29	136	66	1316	1358	1899	2241	2141	1958	2058	1111	1261	1544	1628	1694	1628	1853
43	F	29	105	60	1040	1148	1956	1956	1690	1523	1581	961	919	1227	1794	1569	1361	1361
47	F	29	106	60	1172	1022	1547	2005	2413	2555	2247	1615	1532	1699	1965	2174	2515	2690
48	F	35	110	62	1725	2242	3725	3659	3159	3059	2934	1363	1638	2847	2980	3030	2738	2438
37	F	28	125	60	907	982	1690	1632	1673	1798	1523	1001	1360	1443	1860	1526	1726	1651
38	F	30	150	64	1193	1152	1410	1435	1777	2293	1843	1247	1264	1631	2089	2122	2306	1689
39	F	26	143	63	1261	1053	1511	1645	1578	1745	2111	1029	1137	1237	1679	1596	1737	2012
40	F	27	115	61	1184	1201	1668	2001	1959	1668	1526	1511	1495	2070	2178	2053	1928	2070
				Average	1293	1345	2015	2344	2363	2245	2119	1287	1378	1746	2097	2077	2054	1982

This is contrary to what one would expect on the basis of the findings of Harris and Ludwig ('49) on rats. However, this is not too surprising, as our experience with vitamins A and D leads us to believe that the rat provides an extraordinarily rigorous *in vivo* medium with respect to oxidative activity and a highly efficient *in vivo* hydrolyzing mechanism. We are prone to believe that the conditions for oxidative destruction in the gastrointestinal tract of humans are not nearly so rigorous.

TABLE 2

Comparison of areas under the persistence curves for zero to 24-hour period for 10 male and 10 female subjects

SUBJECT NUMBER	DL-ALPHA-TOCOPHEROL	DL-ALPHA-TOCOPHERYL ACETATE	SUBJECT NUMBER	DL-ALPHA-TOCOPHEROL	DL-ALPHA-TOCOPHERYL ACETATE
	Males			*Females*	
17	10,447	9,677	44	16,669	12,160
18	32,677	26,298	45	38,205	16,151
33	12,378	14,287	46	14,835	12,930
32	9,470	8,757	43	12,156	8,860
30	9,026	9,182	47	24,004	17,849
14	20,166	8,219	48	29,797	27,869
26	14,406	13,939	37	16,146	14,753
31	13,692	8,382	38	16,462	15,832
15	46,924	19,328	39	11,975	16,265
28	25,945	15,046	40	9,832	10,512
Average microgram-hours	19,513	13,312		19,008	15,318

Also, the physiological mechanism affecting the hydrolysis *in vivo* of tocopheryl esters may be less effective in humans than in rats.

If, for example, the blood persistence curve were to be used to measure the occurrence of tocopherol in the blood after dosage with different isomeric forms, one could not claim that the persistence curve provided a measure of relative final physiological effectiveness. However, it has been shown by Engel ('41, '49) and Harris ('51) that the increase in blood tocopherols is entirely in the form of free alpha-tocopherol,

irrespective of whether the free form or the ester is fed. Therefore, in this investigation, the persistence curves may be said to provide a proper measure of relative availability for physiological functions.

One other question occurs: Might the inferior absorption of the alpha-tocopheryl ester indicate a superior usage of the ester in what Hickman ('49) has termed the *in transitu* function of vitamin E? This *in transitu* function in the gastrointestinal tract (as well as later) has been hypothesized by many investigators to be an anti-oxidant function. In view of the fact that free tocopherols have been shown to be superior to tocopherol esters in this respect, the foregoing question can be answered in the negative. The tocopheryl esters, of course, have no anti-oxidant value *in vitro,* and act as anti-oxidants only *in vivo,* after hydrolysis.

SUMMARY

Equivalent, high level dosages of dl-alpha-tocopherol and dl-alpha-tocopheryl acetate were given at suitable intervals to 20 subjects. A series of blood samples were taken and assayed for tocopherol content. The relative utilization of the two forms was determined, using the area between the blood persistence curve and the normal blood level as a measure of absorption.

The utilization of the free alpha-tocopherol was found to be significantly (5% level) superior to the utilization of the alpha-tocopheryl ester. The mean value for the free form was 35% greater than the mean value for the ester.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mrs. Dorothy Rogers for technical assistance in performing the vitamin E analyses.

LITERATURE CITED

EMMERIE, A., AND C. ENGEL 1939 Colorimetric determination of tocopherol (vitamin E). II. Absorption experiments. Rec. trav. chim., *58:* 283.

ENGEL, C. 1941 Splitting of tocopherol phosphoric acid ester *in vivo*. Acta brevia neerl., *11:* 18.

———— 1949 Vitamin E in human nutrition. Ann. N. Y. Acad. Sci., *52:* 292.

HARRIS, P. L. 1951 Personal communication.

HARRIS, P. L., AND M. I. LUDWIG 1949 Vitamin E potency of alpha-tocopherol esters. J. Biol. Chem., *180:* 611.

HICKMAN, K. C. D. 1949 Protective actions of vitamin E in conditions of metabolic stress. Ann. N. Y. Acad. Sci., *52:* 200.

MASON, K. E., AND P. L. HARRIS 1947 Bioassay of vitamin E. Biol. Sympos., Jacques Cattell Press, Lancaster, Pa. Vol. XII, pp. 459.

POPPER, H., AND F. STEIGMANN 1943 On variations of the plasma vitamin A level after the administration of large doses of vitamin A in liver disease. J. Clin. Invest., *XXII:* 775.

QUAIFE, M. L., AND P. L. HARRIS 1944 The chemical estimation of tocopherols in blood plasma. J. Biol. Chem., *156:* 499.

QUAIFE, J. L., W. J. SWANSON, MEI YU DJU AND P. L. HARRIS 1949 Vitamin E in foods and tissues. Ann. N. Y. Acad. Sci., *52:* 300.

SNEDECOR, G. W. 1946 Statistical Methods. The Iowa State College Press, Ames, p. 253.

WEEK, E. F., AND F. J. SEVIGNE 1949a Vitamin A utilization studies. I. The utilization of vitamin A alcohol, vitamin A acetate and vitamin A natural esters by the chick. J. Nutrition, *39:* 233.

———— 1949b Vitamin A utilization studies. II. The utilization of vitamin A alcohol, vitamin A acetate and vitamin A natural esters by the rat. Ibid., *39:* 251.

———— 1950 Vitamin A utilization studies. III. The utilization of vitamin A alcohol, vitamin A acetate and vitamin A natural esters by humans. Ibid., *40:* 563.

VITAMIN A DEFICIENCY IN THE MOUSE [1]

PATRICIA T. McCARTHY AND LEOPOLD R. CERECEDO
Department of Biochemistry, Fordham University, New York, New York

TWO FIGURES

(Received for publication October 10, 1951)

The literature with respect to the status of vitamin A in the rat is extensive both in its scope and in its depth. On the other hand, few investigations into the role of vitamin A in the mouse have been reported. In spite of the many similarities between the two species, their members differ sufficiently to prohibit direct extrapolation of results from one species to another. The increasing importance of the mouse in cancer and in other lines of medical research has rendered an understanding of its nutritional requirements imperative.

Both Groebbels ('24) and Beard ('25) reported that vitamin A is essential for the growth of the mouse. Beard was unable to produce xerophthalmia in mice on his diet, although other symptoms of vitamin A deficiency were present. Spinka ('24) produced xerophthalmia in mice in 50 days and Fujimaki ('26) observed the eye condition in 25 days. In a later paper, Pomerene and Beard ('30) attributed their inability to produce xerophthalmia in mice to the fat component of their diet. By feeding aerated fat they were able to produce ocular symptoms in their mice in 50 to 60 days. Wolfe and Salter ('31) showed that in mice on a vitamin A-free diet the normal epithelium of the various structures was replaced

[1] Presented in part at the April, 1950, meeting of the Federation of American Societies for Experimental Biology in Atlantic City, N. J.

This investigation was supported (in part) by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

by keratinized epithelium in 25 days. The majority of the mice developed xerophthalmia in 90 to 120 days. More recently, in a preliminary report, Fenton, Shrader and Cowgill ('49) described a state of chronic deficiency in three inbred strains of mice placed on a vitamin A-free diet from weaning. The effect of such a diet upon growth rates, organ weights, the blood picture, and the various tissues was described.

The purpose of the present study has been to confirm and to extend the observations of these workers on vitamin A deficiency in the mouse, utilizing highly purified diets containing members of the vitamin B complex in crystalline form. The program conducted in our laboratory included an investigation of the conditions most conducive to the production of a vitamin A deficiency in the mouse, and the characterization of the vitamin A deficiency syndrome in this species by macroscopic and histological studies. The influence of various dietary regimes on the production of the deficiency syndrome, fetal storage of vitamin A, and reproduction function in vitamin A-deficient mice were also investigated.

EXPERIMENTAL AND RESULTS

I. Response of the rat to our vitamin A-deficient diet

For purposes of comparison with the mouse, the effect of the vitamin A-deficient diet designed in our laboratory was first tested on rats with respect to its effect on growth rates, to the time of appearance of the vitamin A deficiency syndrome, to the specific deficiency symptoms and the order of their appearance, and to the life expectancy of vitamin A-deficient rats under the conditions of our experiment. A group of weanling Wistar rats was placed on a vitamin A-free diet of the composition shown in table 1. This diet is a modification of diet R-S-20 devised by Vinson and Cerecedo ('44) and will be designated diet I throughout this study. The rats responded to this regime within 4 to 7 weeks, exhibiting the typical hemorrhagic eye and snout condition. The effect upon the growth rate is shown in figure 1 by comparison with that

of animals receiving ample quantities of vitamin A. The growth rate of vitamin A-deficient rats never approached that of the controls and they began to lose weight at the 11th week of life. The survival time of the deficient rats varied over a range of 18 to 23 weeks.

The immediate response and the reproducibility of results elicited from rats under the dietary regime imposed attested to the relative freedom of our diet from vitamin A, and to the suitability of our experimental conditions for use in vitamin A depletion studies.

TABLE 1

Experimental diets

DIET I		DIET II		SUPPLEMENTS ADDED PER KILOGRAM OF DIET [3]	
	%		%		
Casein	30	Casein	20	Thiamine hydrochloride	20 mg
Sucrose	48	Sucrose	68	Riboflavin	20 mg
Salts [1]	5	Salts [1]	5	Pyridoxine hydrochloride	20 mg
Ruffex	2	Ruffex	2	Calcium pantothenate	40 mg
Hydrogenated vegetable oil [2]	10	Hydrogenated vegetable oil [2]	5	Folic acid	20 mg
Lard	5			Choline chloride	500 mg
				alpha-Tocopherol	20 mg
				Vitamin D (Drisdol)	5,000 units

[1] Osborne-Mendel Salt Mixture.

[2] Crisco.

[3] Controls received, in addition, vitamin A concentrate at a level of 67,500 I.U. per kilogram of diet.

II. Production of a vitamin A deficiency syndrome in mice

Employing the experimental design which was so successful in the rat studies, we attempted to produce a vitamin A deficiency state in mice. In this study three strains of albino mice were used; Swiss-Webster, Rockland, and our own strain, hereafter designated the Fordham strain. These animals were placed upon diet I at weaning. A record was maintained of food intake, consumed ad libitum.

As is shown in figure 1, the growth curves of mice on the vitamin A-free diet paralleled those of the controls. There is no similarity between the curves exhibited by the rats and those of the mice, although both groups of animals were subjected to identical treatment. The length of time required for these mice to develop xerophthalmia varied. Some gave in-

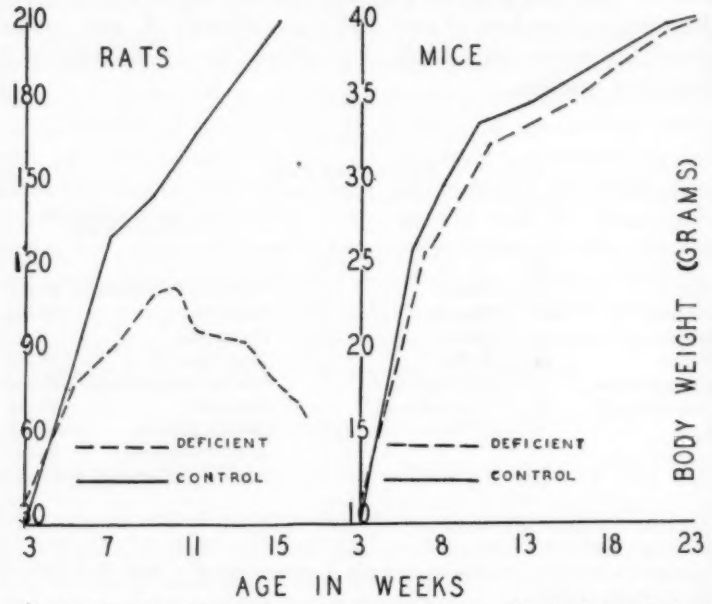

Fig. 1 Growth rates of rats and mice placed upon vitamin A-free diets at weaning.

dications of a deficiency state at 6 months. The majority, however, gave no indications of such a condition before 10 months. Many of these mice, one year or more of age, died before developing ocular symptoms of any kind.

Contrary to experience with the rat, this treatment obviously failed to produce in the mouse a well-defined vitamin A deficiency syndrome within a reasonable length of time. The ability of these mice to withstand long periods of vitamin A

deprivation was attributed to large initial stores at weaning or to a possible sparing effect exerted by the diet employed upon such stores, or both. The superior resistance of the mouse to vitamin A deficiency indicated by these studies suggested the need for more stringent conditions in the production of a clear-cut vitamin A deficiency syndrome in this species. These conditions were investigated both from the point of view of pre-depletion of the initial stores, and from a dietary standpoint.

a. Pre-depletion of the weanling mouse. Pre-depletion of the weanling mouse was accomplished by transferring the mothers on the 11th day of lactation from Rockland stock rations to diet I. The diet when fed at this time had apparently no ill effects upon the ability of the mother to care for her young, nor upon the weaning weights of the young. The young mice were subsequently continued on this vitamin A-free diet at weaning.

The growth curve exhibited by these mice is shown in figure 2. The majority of the animals grew normally for 5 to 6 months. Death occurred at the 10th to the 12th month, and the eye condition appeared at approximately 6 to 10 months of age.

By placing the mothers of the experimental animals on a vitamin A-free diet on the first or second day after parturition, the life span could be significantly reduced, death occurring within two and one-half to 8 months of age, while the deficiency syndrome could be produced within a shorter period of time. The growth curve of these mice is compared in figure 2 with those of mice receiving other pre-depletional treatments.

Since the time of appearance of symptoms and the growth rates could be regulated by varying the lactational background of the weanling with respect to vitamin A, the effect of feeding vitamin A-free diets during gestation was also investigated. Placing the mothers upon such diets during gestation resulted in a further slight decrease in the time of appearance of symptoms. This treatment was unsatisfactory,

however, for obtaining large numbers of experimental animals suitable for further work. The growth curve exhibited by these mice is shown in figure 2.

b. Influence of the diet upon the production of the vitamin A deficiency syndrome in mice. In order to decrease the depletion period, and to secure greater uniformity in the time of appearance of symptoms, different dietary regimes were

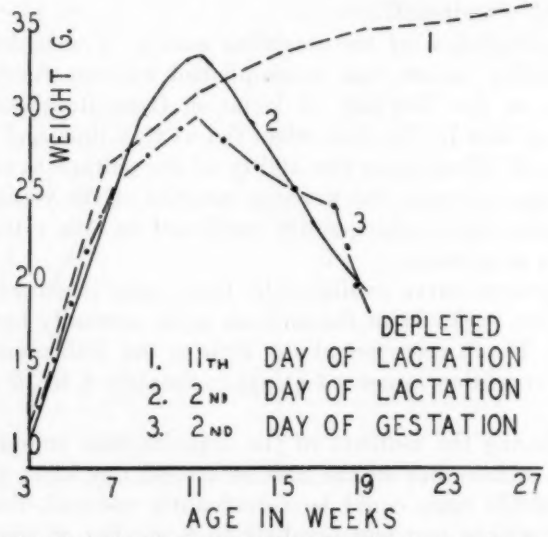

Fig. 2 The effect of various pre-depletional treatments upon the growth rates of vitamin A-deficient mice.

devised and investigated. It has been well-established that there is a definite relationship between dietary components and the production of a vitamin A deficiency. Muelder and Kelly ('42) found that the level of fat in the diet exerted an effect upon the utilization of vitamin A, while Mayer and Krehl ('48) reported that the isocaloric replacement of sucrose with lard resulted in an increase in the survival time and the attainment of larger body weights by rats on vitamin A-deficient diets.

In the present study the casein and fat contents of the diet, diet I, were simultaneously lowered. The most important change, however, was the complete removal of lard, and a reduction in the level of the hydrogenated fat. The complete removal of lard and the reduction of the hydrogenated cottonseed oil [2] and casein contents of the diet were prompted by the desire to decrease to a minimum all possible exogenous sources of vitamin A or of vitamin A precursors. The fat content was also reduced with the purpose of rendering conditions favoring storage and economic utilization of available

TABLE 2

Nutritional background and susceptibility to vitamin A deficiency

DAYS ON VITAMIN A-FREE DIET		DIET AT WEANING	NUMBER [1] OF MICE	TIME OF APPEARANCE OF VITAMIN A DEFICIENCY SYMPTOMS
During gestation	During lactation			
				days
0	0	I	36	180–365
0	4–11	I	25	150–240
0	0	II	24	90–120
0	2	II	20	75–82
0	19	II	22	48–75
0	21	II	35	45–50
5–19	21	II	24	32–52

[1] Data for the three strains are grouped together since there were no significant differences among the strains.

stores of vitamin A more stringent. This modified diet has the composition shown in table 1 (diet II).

The results obtained using this diet as a depletion medium were highly satisfactory. A significant decrease was noted in the time required for mice receiving diet II to exhibit deficiency symptoms as compared with the time when the same symptoms were noted in mice fed diet I (see table 2). When placed on diet II at weaning, the young grew normally during the period of rapid growth and appeared healthy. However, definite deficiency symptoms appeared within 90 to 120

[2] Crisco.

days in 80% of the animals and death occurred after they were 6 months of age. Here again it could be shown that by increasing the length of time spent by the mother on a vitamin A-free diet, the depletion period of the young could be further progressively decreased.

A complete summary of the data showing the relationship between the nutritional background of the mouse and susceptibility to vitamin A deficiency appears in table 2.

On the basis of the findings described above it was possible to devise a set of conditions which led to the production of a clear-cut vitamin A deficiency syndrome in 80% of the animals within 45 to 50 days (table 2). The conditions selected were governed by several prerequisites. First, the method employed must permit the weaning of a large percentage of the viable young born. The weanlings must be relatively healthy, since the use of sickly animals in further experimentation introduces such complications as lowered food consumption, resulting in borderline deficiencies in other nutrients, susceptibility to infections and brief, unpredictable life spans. The vitamin A deficiency syndrome should be definite, clear-cut, and characteristic only of a lack of vitamin A. The depletion period should be as brief as possible, and the appearance of symptoms should occur within a relatively narrow range of time. These prerequisites are adequately fulfilled when a vitamin A deficiency is produced by placing the mothers on diet I at parturition, and transferring the young at weaning to diet II. This treatment insures the weaning of large numbers of relatively healthy mice which will consistently exhibit vitamin A deficiency symptoms within 45 to 50 days of age.

III. Fetal stores of vitamin A in the mouse

The observation that young mice depleted of vitamin A at birth gave responses with respect to weight gains, life spans, and length of time required to deplete the tissues of vitamin A stores which were similar to those of mice born of

mothers depleted during gestation suggested that little vitamin A is transmitted to the fetus during gestation by the mother, and that the young depend upon the maternal source of vitamin A during lactation. From the data presented in table 2 and in figure 2 it would appear that the period of development of the mouse from parturition until weaning is the crucial period with respect to the future ability of the animal to withstand a sustained deprivation of vitamin A initiated at weaning. Furthermore, the results indicated that pregnant females with sufficient vitamin A did not transmit significant quantities of the vitamin for storage purposes to the embryo, and that the stores of this vitamin at birth are meager.

In order to substantiate these deductions made on the basis of the response of young mice to vitamin A-free diets after various forms of pre-depletion, fetal stores of this vitamin were determined at various stages throughout embryonic development, and hepatic stores during lactation. Females of the Swiss-Webster strain, maintained on stock rations, were sacrificed at various intervals during pregnancy, and the embryos removed and analyzed for vitamin A according to the method of Oser et al. ('43). The placentae were removed separately and likewise analyzed for vitamin A.

According to the data presented in table 3, it can be seen that in the mouse, as in many other species, the vitamin A content of the fetus is extremely low. In many cases the negligible quantities present necessitated the use of pooled samples containing as many as 30 embryos in order to obtain detectable amounts of vitamin A.

The vitamin A content of mice sacrificed one to two days after birth showed a striking increase over that of embryos sacrificed just prior to birth. The importance of the lactation period with respect to vitamin A storage is indicated by the tremendous increase in hepatic stores of this vitamin in the weanling. At no time was any vitamin A detected in the placentae.

The presence of small quantities of vitamin A in the embryo indicates that for normal embryonic development the fetus probably needs and utilizes vitamin A. This observation is in line with the work of Warkany and Roth ('48), who observed a wide variety of congenital malformations in the young of female rats reared from weaning to the birth of their young on inadequate levels of vitamin A. The amount of vitamin A stored by the embryo is inadequate, however, for the normal development to adulthood of the young mouse which depends upon the maternal source during lactation.

TABLE 3

Fetal stores of vitamin A

TOTAL NUMBER OF EMBRYOS IN SAMPLE	AGE OF EMBRYOS	AVERAGE VITAMIN A CONTENT PER EMBRYO
	days	*I.U.*
24	14	0.9
5; 12	15	none detected (5); 1.3
15; 15	16	1.6; 1.9
30	15–16	1.3
30	17–18	0.6
13	19	1.3
9 mice	1 day after birth	8.0
6 mice	21 days after birth	122.5
Placentae of 60 embryos	16	none detected

Furthermore, the results obtained in these chemical analyses confirm and explain the results obtained when the biological responses of mice to varying depletion periods were measured.

IV. The effect of fat-free and vitamin E-free diets on the time of appearance of the typical vitamin A deficiency syndrome

Litter mates from a dozen litters of mice of the Swiss-Webster strain were divided into three groups at weaning. The first group received diet II. The second group received diet II from which vitamin E was omitted. The third group

was placed on a fat-free diet, of the basal pattern of diet II, in which the fat was replaced by sucrose. The time of appearance of the eye exudate for each of the three groups was, respectively, 90 to 120 days; 61 to 66 days; and 64 to 80 days. These data show that vitamin E-free and fat-free diets were effective in hastening the onset of the vitamin A deficiency syndrome, the vitamin E-free diet being somewhat more efficacious.

The effect of vitamin E-free and fat-free diets on the rate of depletion of hepatic stores of vitamin A in mice was then investigated. Adult Swiss females from the stock colony, selected because of their relatively large initial stores of

TABLE 4

Effect of vitamin E-free and fat-free diets on rate of depletion of liver stores of vitamin A

TIME ON DEFICIENT DIET	DIET	FINAL BODY WEIGHT	LIVER WEIGHT	TOTAL STORES VITAMIN A	I.U./GM OF LIVER
weeks		*gm*	*gm*	*I.U.*	
0	Stock	45.0	2.86	14,350	5,100
10	II	52.4	3.85	6,750	1,753
10	II less E	44.4	4.05	3,250	800
10	II less fat	31.0	2.85	3,000	1,052

this vitamin, were divided into 4 groups. The first group was killed immediately to determine initial stores. The remaining groups were placed on the three dietary regimes described above. At the end of 10 weeks none of the mice exhibited any symptoms whatsoever of a deficiency state; at this time they were sacrificed and their livers analyzed for vitamin A. The results of this experiment are shown in table 4.

These results indicate that the specific effects of vitamin E-free and fat-free dietary regimes on the time of appearance of vitamin A deficiency symptoms are due to their ability to deplete the liver more rapidly of its stores of vitamin A. The results obtained in liver analyses support the data found in determining the time of appearance of the symptoms.

V. Pathologic changes in vitamin A-deficient mice

Severely vitamin A-deficient mice developed diarrhea, tremors, unkempt fur, and a severe eye condition characterized by a thick, clear, colorless exudate. Unlike the condition we observed in the case of the rat, the eye exudate of the mouse is non-hemorrhagic and there is no nasal exudate. Less characteristic symptoms included abscess formation, distention of the sides, rectal and vaginal hemorrhaging, opaque corneas, and a pronounced inability to stabilize head movements. The addition of vitamin A supplements to the diets of such animals resulted in either a more or less complete alleviation of symptoms or a gain in weight, or both. Damages can, however, be irreversible, in that some animals in an advanced stage did not respond to such therapy.

The most characteristic post mortem finding was that of enlarged kidneys filled with a white opaque mass of material. Such kidneys showed no well-defined structure, only the outer cover remaining intact. Abnormally distended seminal vesicles and thickened urinary bladders filled with the same material, and small, soft testes were characteristic. Abscesses, enlarged salivary glands, stones in the urinary bladder, and hemorrhagic areas on the lungs were less frequent observations. The liver was consistently normal in appearance.

Histologic findings. In agreement with the findings of Wolfe and Salter ('31), histological studies gave a picture similar to that described for the rat. In the present study, sections of the various tissues were kindly examined by Dr. Preston Price of the Jersey City Medical Center. In these mice the pelvic epithelium has been observed to undergo almost complete erosion, and where the pelvic epithelium remains there is squamous metaplasia with marked keratinization. Interstitial foci of lymphocytes within the pyramids and intercapillary hyalinization of the glomeruli were observed only in advanced stages of vitamin A deficiency. Acute pyelonephritis and suggestions of incipient calcification of the pelvic epithelium were noted. The bladder, ureter, vas deferens, semi-

nal vesicles, testes, salivary glands, trachea, and esophagus were lined by well-keratinized squamous epithelium and frequently showed evidence of inflammation. Lumina of the seminal vesicles and salivary gland ducts were filled with keratin, polymorphic cells, and acidophilic material. The testes exhibited interstitial cell hyperplasia, with atrophic spermatic tubules containing no sperm. Changes in the eyes of such animals were far less marked and occurred much later than the marked degeneration of the genito-urinary epithelium. Many showed no change, others merely suggested epithelial changes of the cornea, while a few showed acute

TABLE 5

Data showing the incidence of pathologic changes in vitamin A-deficient mice

CHARACTERISTIC CHANGES		OCCASIONAL CHANGES	NORMAL
Organ	Abnormal		
	%		
Bladder	87	Female reproductive system	Thyroid
Kidney	80	Pancreas	Thymus
Seminal vesicles	66	Salivary glands	Adrenal
Testes	60	Epididymis	Spleen
Trachea	33	Esophagus	Liver
Eye	33	Lung	Heart

conjunctivitis and ulceration. Less frequent observations included interstitial congestion of the lung with a bronchial inflammatory exudate and slight bronchial pneumonia. The relative frequencies with which such changes occurred appear in table 5.

It was of interest to observe that these extensive changes occurred only in those mice allowed to develop the avitaminosis over a long period of time. Mice whose stores of vitamin A were rapidly depleted by placing the mothers on a vitamin A-free diet during gestation or lactation developed an acute avitaminosis and died before attaining 60 days of age. Although a marked eye exudate was observed in these animals, death occurred before structural changes in the eye took place

and before the extensive changes in the epithelium of the various structures of the body described above developed.

Because of the pronounced inability to stabilize head movements shown by many mice, sections were made of the brain and spinal column of a pair of typical mice and were kindly examined by Dr. Abner Wolf of the Columbia University Medical Center. The cerebral cortex, white matter, basal ganglia, cerebellum, brain stem, and spinal cord showed no abnormalities. Degenerative changes in the nervous systems of vitamin A-deficient animals of many species have been reported by Mellanby ('31), Zimmerman ('33), Wolbach and Bessey ('42), and Fletcher and Rigdon ('49). On the basis of our limited investigation, the effect of vitamin A deficiency on the nervous system of the mouse is not at all clear.

VI. The effect of vitamin A deficiency on reproduction

Because of the widespread degeneration of the epithelium of the reproductive system, the effect of vitamin A deficiency on reproduction was investigated. Thirty males and 45 females reared on vitamin A-free diets from weaning were mated to stock colony mice of known fertility. Seven per cent of the vitamin A-deficient males were capable of fertile matings. Fifty-three per cent of the deficient females became pregnant, while only 33% dropped viable litters. Hence, in the female the reproductive function had been impaired but by no means destroyed. The fetuses of those females capable of mating frequently undergo resorption, abortion, intrauterine death, or are still-born. Many females are unable to make normal delivery, dropping their young over a period of several days or dying during the process of delivery. Ectopic pregnancies are frequent. Vitamin A-deficient females capable of bringing their young to term have never been observed to take care of the young after parturition. These young are viable and behave normally when given to foster mothers from the stock colony. In the male, on the other hand, the reproductive function has been virtually destroyed, as is shown by his

inability to impregnate the female. The sterility produced by a deficiency of vitamin A in the male mouse was found to be reversible provided vitamin A therapy was initiated before the degenerative changes had progressed to an advanced state.

SUMMARY

A study of vitamin A deficiency was made in three strains of white mice.

1. Rats and mice depleted of tissue stores of vitamin A under identical conditions differ markedly in their susceptibility to the development of a deficiency state, mice exhibiting greater resistance with respect to weight gains, life spans, and time of appearance of symptoms.

2. A set of conditions was developed which led to the production of a clear-cut vitamin A deficiency syndrome in 80% of the mice within 45 to 50 days. Both the dietary components and the nutritional background of the weanling with respect to vitamin A were all-important in determining the susceptibility of the mouse to an avitaminosis.

3. The small amount of vitamin A transmitted to the embryo by the mother for storage purposes, and the importance of the lactation period as a prime factor in conditioning the mouse's response to vitamin A-deficient diets, were shown.

4. Vitamin E-free and fat-free diets decreased the time required for the appearance of deficiency symptoms on vitamin A-free diets by increasing the rate of depletion of liver stores of vitamin A.

5. Pathologic and histologic changes associated with vitamin A deficiency in the white mouse are described. Abnormalities in the eye were noted only in those animals with an advanced avitaminosis. Eye changes occurred much later than the marked degeneration of the epithelium of the genito-urinary tract.

6. The reproductive function in the vitamin A-deficient male is virtually destroyed, but sterility can be reversed by the addition of vitamin A to the diet. The reproductive function in the vitamin A-deficient female is somewhat impaired

but not completely destroyed. Manifestations of this impaired function are listed.

ACKNOWLEDGMENTS

The cooperation of Dr. Preston Price and Dr. Abner Wolf is gratefully ackowledged.

LITERATURE CITED

BEARD, H. H. 1925 Studies in the nutrition of the white mouse. III. Vitamin factors in the nutrition of the white mouse. Am. J. Physiol., *75:* 668.

FENTON, P. F., R. E. SHRADER AND G. R. COWGILL 1949 Chronic vitamin A deficiency in three inbred strains of mice. Fed. Proc., *8:* 382.

FLETCHER, D. E., AND R. H. RIGDON 1949 Neurologic manifestations associated with vitamin A deficiency in the young duck. Arch. Neur. Psychiat., *61:* 199.

FUJIMAKI, Y. 1926 The formation of urinary and bile duct calculi in animals fed on experimental rations. Japan Med. World, *6:* 29.

GROEBBELS, F. 1924 Studien über das Vitaminproblem. III. Weitere Untersuchungen über den Einfluss der Vitaminzufuhr auf Gasstoffwechsel, Gewicht und Lebensdauer vitaminfrei ernährter weisser Mäuse. Zschr. physiol. Chem., *137:* 14.

MAYER, J., AND W. A. KREHL 1948 The relation of diet composition and vitamin C to vitamin A deficiency. J. Nutrition, *35:* 523.

MELLANBY, E. 1931 Experimental production and prevention of degeneration in the spinal cord. Brain, *54:* 247.

MUELDER, K. D., AND E. KELLY 1942 The effect of the level of fat in the diet upon utilization of vitamin A. J. Nutrition, *23:* 335.

OSER, B. L., D. MELNICK AND M. PADER 1943 Estimation of vitamin A in food products. Ind. Eng. Chem., Anal. Ed., *15:* 724.

POMERENE, E., AND H. H. BEARD 1930 Studies in the nutrition of the white mouse. VI. The experimental production of xerophthalmia in mice. Am. J. Physiol., *92:* 282.

SPINKA, J. 1924 Untersuchungen über den Einfluss ultravioletter Strahlen auf akzessorische Stoffe. Biochem. Zschr., *153:* 197.

VINSON, L. J., AND L. R. CERECEDO 1944 Growth, reproduction and lactation in mice maintained through four generations on highly purified diets. Arch. Biochem., *3:* 389.

WARKANY, J., AND C. B. ROTH 1948 Congenital malformations induced in rats by maternal vitamin A deficiency. J. Nutrition, *35:* 1.

WOLBACH, S. B., AND O. A. BESSEY 1942 Tissue changes in vitamin deficiencies. Physiol. Rev., *22:* 233.

WOLFE, J. M., AND H. P. SALTER 1931 Vitamin A deficiency in the albino mouse. J. Nutrition, *4:* 185.

ZIMMERMAN, H. M. 1933 Lesions of the nervous system in vitamin deficiency. I. Rats on a diet low in vitamin A. Am. J. Exp. Med., *57:* 215.

SOYBEAN LECITHINS AND VITAMIN A UTILIZATION [1]

N. B. GUERRANT AND R. Q. THOMPSON

Department of Agricultural and Biological Chemistry, The Pennsylvania State College, State College

ONE FIGURE

(Received for publication October 1, 1951)

The published results of a number of investigations have indicated that the efficiency of vitamin A utilization by the rat is dependent on several factors, some of which are of dietary origin. Among the factors of dietary origin which seem to have the greatest influence on the efficiency of vitamin A utilization are: (a) the fat content of the diet, (b) the dosage of vitamin A fed, and (c) the tocopherol content of the vitamin A carrier and perhaps of the fat component of the basal diet. However, in recent years there have appeared a number of reports which indicate that the addition of lecithins to the diet of the rat results in a significant increase in the efficiency of vitamin A utilization and storage. This seems particularly true of the crude soybean lecithin (Esh and Sutton, '48; Scharf and Slanetz, '44; Slanetz and Scharf, '43, '45). Furthermore, crude soybean lecithin also appears to influence vitamin A utilization by human subjects (Adlersberg and Sabotka, '43) and by cattle (Esh et al., '47, '48).

Some investigators have attributed this enhancing property of crude soybean lecithin to a contaminant, while others have been inclined to attribute the unusual biological property to the characteristics of the lecithin molecule. Since earlier

[1] Authorized for publication on September 25, 1951, as paper 1690 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

studies with rats subsisting on the U.S.P. vitamin A-deficient diet supplemented with limited amounts of highly purified animal and plant lecithin, and eventually supplemented also with small daily intakes of vitamin A, did not yield data which would suggest a synergistic effect as being due to these materials, a study of crude soybean lecithin under comparable conditions seemed worthy of consideration.

Furthermore, it seemed reasonable to suspect that if soybean lecithin does exert a synergistic effect on vitamin A utilization, this effect would be most pronounced under experimental conditions in which slight differences in vitamin A utilization are readily detected; namely, under the conditions of the U.S.P. bioassay (Pharmacopoeia of the United States, '50).

EXPERIMENTAL

In the initial studies in this investigation, two types of soybean lecithin were used.[2] One was an unbleached plastic product and the other was a similar product but had been bleached to improve its color. The former (unbleached) was a chocolate-brown waxy material, while the latter (bleached) was a product of similar consistency but was considerably lighter in color. When preliminary studies with the above lecithins resulted in some unexpected observations, two additional samples of soybean lecithin were obtained from another source.[3] Both of the latter products were dark brown in color and were heavy, oily liquids. They appeared to be commercial soybean lecithin dispersed in vegetable oil. When received, one of the products bore the label "Control 2 Lecithin," while the other product bore the label "Control 4 Lecithin." For the purpose of the present report the first two products have been designated "unbleached" and "bleached" lecithins, respectively, and the latter two products as "C-2" and "C-4" lecithin, respectively. In addition to the above soybean lecithins, a quantity of purified vege-

[2] Obtained through the courtesy of Dr. J. S. Andrews, General Mills, Inc., Minneapolis, Minn.

[3] Purchased from the Central Soya Company, Inc., Fort Wayne, Ind.

table lecithin was purchased for use in the studies.[4] All lecithins were stored under refrigeration and in the original containers until used.

In the early phase of the studies comparable groups of undepleted rats were fed portions of the U.S.P. vitamin A-deficient diet into which 5% of the respective lecithins had been incorporated at the expense of the carbohydrate component. When the rats reached the critical state of depletion, each received a vitamin A supplement (in the form of the U.S.P. Vitamin A Reference Standard) during a subsequent 28-day period.

When it became evident that the presence of 5% of certain of the lecithins in the basal diet resulted in an extended depletion period, other studies were initiated in which the rats were first depleted of their vitamin A reserves before receiving the lecithin supplement. In the latter studies, groups of depleted rats were fed portions of the vitamin A-deficient diet into which had been incorporated definite amounts of the respective lecithins. Other groups of depleted rats received the deficient diet supplemented with concentrates of the non-saponifiable residues from definite amounts of the respective lecithins. The saponifications and extractions were carried out essentially in accordance with the official method (Association of Official Agricultural Chemists, '50), except that larger portions of samples and of reagents were involved and that petroleum ether was used as the extracting solvent for the non-saponifiable residues instead of diethyl ether. Still other groups of depleted rats received the deficient diet into which had been incorporated, respectively, the acetone-soluble portion of the lecithins, the acetone-insoluble portion of the lecithins, purified vegetable lecithin, hydrogenated cottonseed oil, inositol, and choline. In addition, certain of these groups of rats and other groups of depleted animals received supplements of known amounts of vitamin A. All supplementary feeding was continued over a 28-day period, or until the test animal succumbed. The general plan of the supple-

[4] Purchased from the Nutritional Biochemical Corp., Cleveland, Ohio.

mentary feedings and the average responses of each of the several groups of rats are indicated by the data presented in table 1.

DISCUSSION

It is to be noted from the data presented in table 1 that when certain of the soybean lecithins were incorporated in the U.S.P. vitamin A-deficient diet at the rate of 5% and the mixture fed to groups of normal young rats (groups 1 to 4), the time required to deplete these rats of their body stores of vitamin A was extended by several days. Furthermore, it is to be noted that when the depleted rats were given a daily supplement of 2.0 U.S.P. units of vitamin A, their growth responses were somewhat greater than those of a comparable group of rats receiving the 2.0 units of vitamin A and a 5% supplement of purified plant lecithin (group 5), or those of rats receiving the 2.0 units of vitamin A without a lecithin supplement (group 8). These observations suggest that either the crude soybean lecithins were exerting a synergistic effect on vitamin A economy or these preparations were supplying a measurable amount of a biologically active material.

To investigate these possibilities, a series of feeding tests were carried out in which the diets of groups of vitamin A-depleted rats were supplemented with the various soybean lecithins, with the respective non-saponifiable residues of these lecithins, and with other fractions prepared from the crude lecithins. These lecithin additions to the diets were made without and with vitamin A supplementation.

When groups of vitamin A-depleted rats received the diets containing 5% of each of the crude soybean lecithins, those rats which received the diets containing the "unbleached" (group 11) and the "bleached" (group 15) lecithins responded and made positive but irregular gains in weight during the 28-day test period; whereas those rats which received the "C-2" (group 20) and the "C-4" (group 23) lecithin supplements continued to lose weight, although neither the weight loss nor the mortality was as severe as that which occurred among the rats comprising the negative control group (group

6). When diets containing 10% of the "unbleached" and the "bleached" lecithins were fed (groups 10 and 14), the depleted rats resumed growth and grew at a rate which exceeded that of comparable rats receiving 1.5 U.S.P. units of vitamin A daily (group 7). Since the rats consumed approximately 0.9 gm of the lecithins daily, this would indicate that 1 gm of these lecithins exerted the biological activity of approximately 1.5 to 2.0 units of vitamin A. Depleted rats which received daily supplements of the non-saponifiable residues of the respective lecithins (groups 12, 16, 21, 25) made less gain in weight than did those animals receiving the corresponding unsaponified lecithins, but the results did serve to demonstrate that the greater portion of the biologically active material was passing into the non-saponifiable residues instead of remaining with the soaps.

The most active of the lecithins ("bleached") was fractionated by acetone extraction, the acetone removed by distilling under reduced pressure, the two fractions (acetone-soluble and acetone-insoluble) incorporated in the vitamin A-deficient diet at a rate equivalent to 10% of the original lecithin, and the mixtures fed to groups of depleted animals (groups 18 and 19). Those rats which received the acetone-soluble fraction made approximately 70% as much growth during the 28-day supplementary period as did comparable rats receiving an equivalent percentage of the unfractionated lecithin. On the other hand, 6 of the 8 rats which received the acetone-insoluble fraction survived the experimental period, but each lost weight during it.

When the lecithin-containing diets (5%) were further supplemented with 2.0 units of vitamin A daily, the responses of the test animals were invariably favorable. Mortality was insignificant and the growth rate for most groups of animals exceeded that of comparable groups of rats receiving only the vitamin A supplement (group 8). However, the increase in growth could be accounted for on an additive basis without attributing synergistic properties to the lecithins.

TABLE 1

Effect of soybean lecithin on vitamin A utilization by the rat as measured by depletion time and by food intake and weight change during a 28-day period of supplementation

EXPERIMENTAL GROUP	SUPPLEMENTS ADDED TO THE VITAMIN A-DEFICIENT DIET	DEPLETION PERIOD			SUPPLEMENTARY PERIOD			
		Ave. initial wt.	Ave. depletion time	Ave. wt. of depleted rat	No. of rats used	No. of rats surviving	Total[1] food consumed	Ave. wt. change
		gm	*days*	*gm*			*gm*	*gm*
	Studies with undepleted rats[2]							
1	5% of Unbleached lecithin	45.0	35.6	135	8	8	260	44.5
2	5% of Bleached lecithin	44.2	37.0	134	8	8	246	46.4
3	5% of (C-2) Lecithin	44.6	33.2	128	8	8	230	39.8
4	5% of (C-4) Lecithin	44.1	31.9	124	8	8	239	39.2
5	5% of Purified vegetable lecithin	43.9	30.4	118	8	8	228	38.9
	Studies with depleted rats[3]							
6	None (negative control)	44.9	30.1	116	22	4	109	— 39.3
7	1.5 U.S.P. units vitamin A daily	44.0	28.9	116	19	19	220	31.3
8	2.0 U.S.P. units vitamin A daily	44.2	29.5	111	57	56	222	40.1
9	2.5 U.S.P. units vitamin A daily	44.0	29.1	107	17	17	246	52.6
10	10% Unbleached lecithin	44.1	32.0	113	16	16	206	35.3
11	5% Unbleached lecithin	44.3	29.6	114	12	12	172	8.2
12	Non-sap. residue of unbleached lecithin equal to 5%	44.0	29.0	111	12	10	160	5.9
13	5% of Unbleached lecithin + 2.0 units vitamin A daily	45.0	30.6	117	14	14	221	46.7

10	10% Unbleached lecithin	44.1	32.0	113	16	16	206	35.3
11	5% Unbleached lecithin	44.3	29.6	114	12	12	172	8.2
12	Non-sap. residue of unbleached lecithin equal to 5%	44.0	29.0	111	12	10	160	5.9
13	5% of Unbleached lecithin + 2.0 units vitamin A daily	45.0	30.6	117	14	14	221	46.7
14	10% of Bleached lecithin	44.2	30.9	116	8	8	204	38.1
15	5% of Bleached lecithin	43.8	29.7	113	12	12	192	11.4
16	Non-sap. residue of bleached lecithin equal to 5%	43.5	29.2	113	12	12	177	9.1
17	5% of Bleached lecithin + 2.0 units vitamin A daily	45.6	30.1	115	12	12	224	46.5
18	Acetone-insoluble portion of bleached lecithin equal to 10%	45.6	29.7	121	8	6	164	— 20.8
19	Acetone-soluble portion of bleached lecithin equal to 10%	46.5	31.0	122	8	8	234	26.6
20	5% of (C-2) Lecithin	46.1	29.9	113	20	13	126	— 22.9
21	Non-sap. residue of (C-2) lecithin equal to 5%	43.0	30.1	115	8	3	135	— 29.6
22	5% of (C-2) Lecithin + 2.0 units of vitamin A daily	44.5	29.0	114	16	16	218	42.1
23	5% of (C-4) Lecithin	42.7	29.5	110	19	15	142	— 23.3
24	5% of (C-4) Lecithin + 2.0 units of vitamin A daily	44.4	29.2	116	16	16	218	41.8
25	Non-sap. residue of (C-4) lecithin equal to 5%	44.0	29.0	114	5	4	152	— 20.4
26	Non-sap. residue of (C-4) lecithin equal to 5% + 2.0 units vitamin A daily	44.1	28.5	113	5	5	227	39.2
27	5% Hydrogenated cottonseed oil + 2.0 units vitamin A daily	43.3	31.2	114	9	9	208	42.8
28	0.1% Inositol + 1.5 units vitamin A daily	45.5	28.1	122	10	10	227	33.5
29	0.1% Choline + 1.5 units vitamin A daily	44.1	28.0	118	10	10	226	30.0
30	1.5 Units vitamin A daily containing 15 mg purified plant lecithin	44.2	29.0	118	9	9	223	31.6

[1] Average weight of food consumed during the 28-day period of supplementation.

[2] The lecithin was incorporated in the vitamin A-deficient diet at the beginning of the feeding period. When the rats were depleted of their vitamin A reserve, they received the equivalent of 2.0 U.S.P. units of vitamin A daily for a 28-day test period. The U.S.P. Reference Standard was used as the source of supplementary vitamin A.

[3] The rats were depleted of their vitamin A reserve before any supplement was fed.

Inasmuch as it had been suggested (Adlersberg and Sabotka, '43) that crude soybean lecithin may exert its "vitamin A-sparing effect" through the presence of such chemical structures as inositol or choline, groups of depleted rats were fed diets containing 0.1% of inositol and of choline, respectively, and the diets were further supplemented with vitamin A at the rate of 1.5 U.S.P. units per rat per day (groups 28 and 29). The resulting growth responses were unchanged as the result of the inositol or choline supplementation. Likewise, the addition of purified vegetable lecithin to the vitamin A standard [5] at the rate of 150 mg per milliliter resulted in no perceptible increase in the rate of growth of vitamin A-depleted rats over that produced by vitamin A alone.

On the basis of the above studies it seemed evident that the substance or substances present in different batches of commercial soybean lecithins in varying amounts and which, when incorporated in the vitamin A-deficient diet, resulted in a delay in the rate of depletion of the rat, and when fed to the depleted rat in sufficient amounts caused a resumption of growth, is not lecithin, nor perhaps one of the degradation products of lecithin, unless the specific product is soluble in acetone and in petrolic ether and will withstand rather vigorous saponification.

In an attempt to identify some of the constituents of the non-saponifiable residue from the "bleached" lecithin through chromatography and spectrography, it was observed that the alkali-free concentrate, when dissolved in petrolic ether and subjected to spectrophotometric examination, yielded an absorption pattern resembling that of mixed carotenoids. When this non-saponifiable material was chromatographed on dicalcium phosphate, the petrolic ether eluate yielded an absorption pattern remarkably similar to that of beta carotene under comparable measurement conditions (fig. 1), although the amount of pigment present in the eluate was a relatively small fraction of the total color present in the non-saponi-

[5] Vitamin A acetate in Wesson oil, 15 units per milliliter.

fiable residue. Furthermore, biological tests on the chromatographed pigment showed it to have growth restorative properties when fed to vitamin A-depleted rats.

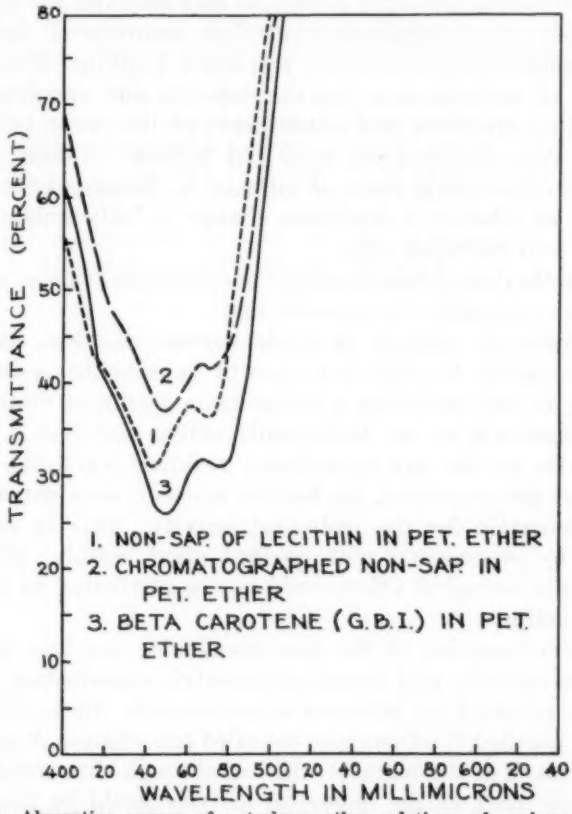

Fig. 1 Absorption curves of petroleum ether solutions of soybean lecithin concentrates and of crystalline beta carotene as determined by the General Electric Recording Spectrograph.

SUMMARY

Studies were made relative to the influence of commercial soybean lecithins on vitamin A utilization by the rat. Four samples of crude soybean lecithins and one sample of purified

plant lecithin were used in the studies. Groups of normal rats and groups of vitamin A-deficient rats were fed a vitamin A-deficient diet containing definite percentages of the crude lecithins, and other groups of rats received the vitamin A-deficient diet containing equivalent amounts of the non-saponifiable residues from the respective lecithins. Still other groups of animals were fed the deficient diet supplemented with other fractions and constituents of the crude lecithins. The lecithin supplements were fed without vitamin A and with restricted daily doses of vitamin A. Records were made of rate of vitamin A depletion, change in body weight, food intake, and mortality rate.

From the data obtained under the conditions of these studies it seems reasonable to conclude that:

1. Different samples of crude soybean lecithins vary in their capacity to stimulate growth in vitamin A-deficient rats or in rats receiving a suboptimum intake of vitamin A.

2. Inasmuch as the biologically active material is found primarily in the non-saponifiable residues when the crude lecithins are saponified, the lecithin molecule does not seem to be responsible for the biological activity. This is substantiated by experiments with purified plant lecithin. Furthermore, the biological effect could not be attributed to inositol or to choline.

3. Fractionation of the non-saponifiable residues through chromatography and spectrophotometric examination of the eluates revealed the presence of carotenoids. Biological tests on the purified fractions also revealed pro-vitamin A activity. While much of the pigment was lost through chromatography and only part of the biological activity could be accounted for in the final eluate, it seems reasonable to conclude that the "vitamin A-sparing effect" of commercial soybean lecithins is due at least in part to the presence of carotenoids.

LITERATURE CITED

ADLERSBERG, D., AND H. SABOTKA 1943 Influence of lecithin feeding on fat and vitamin A absorption in man. J. Nutrition, *25:* 255.

Association of Official Agricultural Chemists 1950 Official Methods of Analysis. Page 767, 7th ed.

ESH, G. C., AND T. S. SUTTON 1948 The effects of soya lecithin on the absorption, utilization, and storage of vitamin A and carotene in the white rat. J. Nutrition, *36:* 391.

ESH, G. C., T. S. SUTTON, J. W. HIBBS AND W. E. KRAUSS 1947 The effect of soya lecithin on the absorption and utilization of vitamin A. J. Animal Sci., *6:* 485.

———— 1948 Effect of soya phosphatides on the absorption and utilization of vitamin A in dairy animals. J. Dairy Sci., *31:* 461.

Pharmacopoeia of the United States 1950 Page 789, 14th rev.

SCHARF, A., AND C. A. SLANETZ 1944 Effect of the soybean phosphatides on the utilization of the fat-soluble vitamins. Proc. Soc. Exp. Biol. Med., *57:* 159.

SLANETZ, C. A., AND A. SCHARF 1943 The effect of phosphatides on the utilization of vitamin A and carotene. Ibid., *53:* 17.

———— 1945 Effect of soybean phosphatides on vitamin A metabolism. J. Nutrition, *30:* 239.

EFFECT OF VITAMIN B_{12} AND FOLIC ACID ON GROWTH AND URICEMIA OF CHICKENS FED HIGH LEVELS OF GLYCINE[1, 2]

L. J. MACHLIN, A. H. LANKENAU, C. A. DENTON AND H. R. BIRD
Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville, Maryland

(Received for publication October 4, 1951)

High levels of protein have been found to inhibit the growth of rats (Cary et al., '46; Hartman et al., '49; Bosshardt et al., '50) and chickens (Rubin and Bird, '47) fed diets deficient in vitamin B_{12}. This finding indicated that the vitamin has a function in the metabolism of protein. Several reports have since substantiated this hypothesis. An elevated non-protein nitrogen level in the blood of chickens fed a diet deficient in the "animal protein factor" (McGinnis et al., '48) and of rats fed "zoopherin"-deficient rations (Zucker and Zucker, '48) has been demonstrated. Subsequent work indicates that this effect was probably due to a deficiency of vitamin B_{12}.

Charkey et al. ('50) have reported that the ingestion of B_{12} by growing chickens deficient in this vitamin reduces the level of amino acids in the blood. They postulated that the vitamin functions by enhancing the utilization of circulating amino acids for the building of tissues. Liener and Schultze ('50) have indicated that the acute uremia observed in newborn rats from mothers maintained on all-plant rations may be due to a failure to utilize absorbed amino acids for protein anabolism. Administration of vitamin B_{12} prevented the uremia.

[1] Report of a study made under the Research and Marketing Act of 1946.

[2] Presented at the 32nd annual meeting of the American Institute of Nutrition, Cleveland, Ohio, May, 1951.

The purpose of the present study was to determine more precisely the nature of the role of vitamin B_{12} in protein metabolism. In order to determine whether B_{12} would overcome the inhibitory effects of a single amino acid, glycine was fed at different levels to B_{12}-deficient and B_{12}-replete chickens. Since uric acid is the primary end product of amino acid catabolism in the chicken, the blood uric acid level was observed in most experiments. Furthermore, as there have been several reports that folic acid alleviates the toxic effects of high levels of glycine in the diet of rats (Dinning et al., '49; Kelly et al., '50; Pagé et al., '49b), the effect of this vitamin on glycine toxicity in growing chickens was also investigated.

While the work was in progress, Menge and Combs ('50) reported that a vitamin B_{12} concentrate overcame the inhibitory effects of 1 and 4% glycine in the diet of chickens. More recently Stern and McGinnis ('51) demonstrated the alleviation by vitamin B_{12} of the toxicity produced by administering a 1 gm portion of glycine by capsule to growing chickens.

EXPERIMENTAL

Rhode Island Red chicks from dams fed a diet deficient in vitamin B_{12} were used in 4 experiments to determine the effects of the following vitamins on glycine toxicity: experiment 1, vitamin B_{12}; experiment 2, B_{12}, folic acid and choline; experiments 3 and 4, B_{12} and folic acid. The effect of these vitamins and glycine on the level of uric acid in the blood was investigated.

For each experiment, day-old chicks were divided into groups of 20 birds each and equalized as to weight distribution. Feed and water were supplied ad libitum and records of weekly weights, mortality, and deficiency symptoms were kept. The birds were reared under electrically heated brooders in wire-floored cages.

At the end of the experiments blood samples were collected after decapitation of the birds for uric acid determinations. When possible, blood was obtained from 10 chicks in each

group, and in all cases the samples were pooled according to experimental treatment. Serum was prepared for analysis in the first experiment but in subsequent experiments plasma was used. The method described by Brown ('45) was used for uric acid determinations.

A corn-soybean oil meal diet was fed in the first three experiments. This consisted of: ground yellow corn 58.0%, alfalfa leaf meal 3.0%, soybean oil meal 35.0%, butyl fermentation solubles 0.6%, tricalcium phosphate 1.4%, limestone 1.0%,

TABLE 1

Synthetic diet used in experiment 4

INGREDIENTS	AMOUNT	VITAMINS ADDED	
	%		mg/kg
Glucose (cerelose)	66.7	Thiamine	10.0
Alpha soybean protein	23.0	Riboflavin	10.0
Wesson oil	3.0	Calcium pantothenate	20.0
Fish oil (4,000 A–400 D)	0.5	Pyridoxine-HCl	6.0
DL-methionine	0.6	Niacin	50.0
L-leucine	0.2	Para-aminobenzoic acid	2.0
Mineral mix 1 M[1]	6.0	2-Methyl-1,4-naphthoquinone	5.0
		Biotin	0.2
		α-Tocopherol	0.5
		Choline-HCl	2,000.0
		Inositol	1,000.0

[1] As reported by Briggs ('46). As a further source of phosphorus, 5.8 ml of H_3PO_4 were added per 100 kg of this diet.

salt (containing 4% manganese sulphate) 0.7%, vitamins A and D in oil 0.2%, DL-methionine 0.1%, and 10 mg of niacin per kilogram of diet.

The effect of the addition of glycine, vitamin B_{12}, folic acid and choline was tested on this diet or a modification of it. Glycine was fed in varying quantities and replaced equal quantities of corn in all cases. Since the basal diet described above supplied appreciable quantities of folic acid, to permit a more effective study of the action of this vitamin a synthetic diet was fed in experiment 4. This diet is given in table 1. Thiamine was administered by dropper weekly, since this

vitamin is destroyed in the presence of alpha protein (Menge et al., '51).

In experiments 3 and 4 the kidneys and livers of several birds from each group were removed for histological examination. Sections were stained with Delafield's hematoxylin and counterstained with eosin Y.

RESULTS

Growth and mortality

In experiment 1 (table 2) all levels of glycine were toxic to the chickens fed the basal diet, as is shown by the effect on

TABLE 2

Experiment 1 — effect of vitamin B_{12} on glycine toxicity

AMOUNT OF VITAMIN B_{12} [1] FED	ADDED GLYCINE	AVERAGE WEIGHT AT 6 WK.	MORTALITY	URIC ACID IN SERUM
μg/kg	%	*gm*	*number dead*	*mg %*
None	0	427	6	3.3
	3	366	8	2.6
	6	269	8	4.9
	9	222	12	5.6
40	0	468	2	4.8
	3	456	1	3.3
	6	456	4	5.4
	9	298	3	9.7

[1] Crystalline vitamin B_{12} — Merck, 0.1% NaCl triturate.

growth, whereas on the B_{12}-supplemented diets only the 9% level of glycine had a significant deleterious effect on growth. Vitamin B_{12} prevented the high mortality caused by the 9% level of glycine, however.

The supplements fed and the results obtained in experiment 2 are given in table 3. There was apparently no vitamin B_{12} or folic acid deficiency in chickens fed the basal diet, since there was almost no mortality and the birds grew as well as those receiving these supplements. The lack of response to B_{12} was probably due to the fact that these chickens

had not been sufficiently depleted of the vitamin. In the presence of 6% glycine, however, there was a significant growth response due to the addition of B_{12}. Folic acid and choline did not stimulate growth individually under these conditions; however, when both were added, growth was increased.

The effect of both vitamin B_{12} and folic acid on growth and mortality was confirmed in the third experiment. The basal diet was modified by omitting alfalfa meal and butyl fermentation solubles, reducing the soybean meal to 30%, and adding the following, in milligrams per kilogram of diet:

TABLE 3

Experiment 2 — effect of vitamin B_{12}, folic acid, and choline on glycine toxicity

SUPPLEMENT	AVERAGE WEIGHT AT 5 WKS.	MORTALITY
	gm	*number dead*
None	364	1
B_{12} [1]	366	0
Choline [2] and folic acid [3]	339	4
6% glycine	286	6
6% glycine + B_{12}	337	5
6% glycine + folic acid	299	3
6% glycine + choline	264	7
6% glycine + folic acid and choline	325	2

[1] Forty micrograms per kilogram.
[2] One thousand milligrams per kilogram.
[3] Two milligrams per kilogram.

choline 1,000, niacin 20, riboflavin 6, and calcium pantothenate 6. This reduced the folic acid to approximately 2.5 mg per kilogram. There was an increase in mortality and a pronounced growth depression when glycine was added to the basal diet (table 4). Folic acid lessened the mortality and helped to overcome the growth depression, although not as much as did B_{12}. As folic acid gave no growth response when added to the B_{12}-supplemented basal diet, it appears that the additional glycine increased the folic acid requirement.

In the 4th experiment a synthetic diet supplemented with 6% glycine was used. The results are given in table 5. Large

quantities of folic acid failed to elicit a growth response as great as that induced by the addition of B_{12}. With B_{12} and the highest level of folic acid, the growth and mortality of the chickens were comparable to those of the birds receiving no additional glycine in the diet. Folic acid and B_{12} together were more effective than either alone in overcoming the growth-depressing effect of glycine.

In all experiments chickens on diets containing 6 or 9% glycine showed the enlargement of the eyeballs previously

TABLE 4

Experiment 3 — effect of vitamin B_{12} and folic acid on glycine toxicity and on the level of uric acid in plasma

SUPPLEMENT	AVERAGE WEIGHT AT 4 WKS.	MORTALITY	URIC ACID IN PLASMA
	gm	*number dead*	*mg %*
None	170	4	4.0
B_{12} [1]	226	4	5.5
Folic acid [2]	197	1	4.2
B_{12} and folic acid	219	1	4.0
6% glycine	99	12	8.6
6% glycine + B_{12}	172	4	10.2
6% glycine + folic acid	142	4	6.1
6% glycine + folic acid + B_{12}	206	3	6.5

[1] Forty micrograms per kilogram.
[2] Four milligrams per kilogram.

described by Groschke et al. ('48). Neither vitamin B_{12} nor folic acid had any marked effect on this syndrome, but they did relieve the prostration and leg weakness caused by high-glycine diets.

Histological examination revealed no obvious changes in the kidneys and livers caused by the experimental treatments.

Level of uric acid in the blood

There was some variation among similar groups in uric acid levels between experiments. This was probably due to differences in diet and degree of depletion of vitamin B_{12}

in the chickens used. However, it is evident from tables 2, 4, and 5 that glycine fed at levels of 6 and 9% caused an elevation of uric acid levels in the blood of growing chickens. The 3% level in the first experiment appeared to decrease blood uric acid somewhat. In all experiments the uric acid content of the blood was raised by the addition of vitamin B_{12} to the basal diets. In experiment 1, B_{12} also increased the uric acid content in all the glycine-supplemented groups. A chick in the group receiving 9% glycine and B_{12} had large

TABLE 5

Experiment 4 — effect of B_{12} and folic acid on glycine toxicity and on the level of uric acid in plasma

SUPPLEMENT	LEVEL OF FOLIC ACID	WEIGHT AT 4 WKS.	MORTALITY	URIC ACID IN PLASMA
	mg/kg diet	*gm*	*number dead*	*mg %*
None	2	158	1	4.5
B_{12} [1]	2	211	2	6.5
6% glycine [2]	0	113	12	15.2
6% glycine	2	148	9	10.1
6% glycine	10	134	10	9.2
6% glycine	50	141	16	4.3
6% glycine + B_{12}	0	131	6	14.7
6% glycine + B_{12}	2	177	3	9.5
6% glycine + B_{12}	10	167	6	..
6% glycine + B_{12}	50	196	4	10.4

[1] Forty micrograms crystalline B_{12} per kilogram of diet.

[2] Replaced cerelose.

deposits of urates in its feet. The condition looked similar to that observed by Lloyd et al. ('49) in turkeys fed diets high in protein. Other chicks in this group showed some lameness and swelling in the joints.

In experiment 3, among groups receiving glycine or B_{12} or both, the uric acid level in the blood was lower in the folic acid-supplemented groups than in those groups not receiving folic acid.

In experiment 4, those chickens receiving glycine and no folic acid had a marked uricemia. Addition of 2 mg/kg of

folic acid caused a decrease in uric acid in both B_{12}-deficient and B_{12}-supplemented groups. A massive dose of folic acid (50 mg/kg) depressed further the uric acid levels of those birds not receiving B_{12} in their diet.

DISCUSSION

These experiments confirm reports (Menge and Combs, '50; Stern and McGinnis, '51) of a decrease in glycine toxicity in growing chickens brought about by the administration of vitamin B_{12}. They also indicate that folic acid is concerned in the metabolism of high levels of glycine in these chickens. It is possible that at least part of the effect of the glycine is to alter the intestinal flora in such a way as to lessen the synthesis or increase the destruction of these vitamins. Pagé and Gingras ('49a) suggested that there is reduced intestinal synthesis of some vitamins in rats due to prolonged feeding of glycine. Since Stern and McGinnis ('51) obtained a toxic condition only 24 hours after administration of glycine, which was counteracted by vitamin B_{12}, glycine toxicity probably cannot be explained solely on the basis of a changed intestinal flora.

An increase in the uric acid level of the blood due to glycine supplementation was expected, since the nitrogen of excess amino acids in growing chickens is excreted chiefly as uric acid. Moreover, glycine has been shown to be a direct precursor of uric acid in birds (Buchanan et al., '48; Karlsson and Barker, '49) and can evidently be utilized intact for uric acid synthesis.

The exact mechanism of the effect of vitamin B_{12} and folic acid on the uric acid levels cannot be stated at this time. They may act on renal mechanisms, although in the present work no evident kidney damage was found in any of the groups examined.

There was no correlation between the level of uric acid in the blood of chickens and their growth. In experiments 3 and 4 uric acid levels were sometimes reduced by folic acid,

although there was no increase in growth. Conversely, the level of uric acid in the blood was increased by vitamin B_{12}, although there was usually an increase in growth. Therefore, the change in blood uric acid level is not an indication of increased or decreased utilization of amino acids for tissue growth. It is possible that the lowering of high uric acid levels by folic acid is due to direct inhibition of uric acid synthesis, or it may be merely an indirect effect resulting from better utilization of glycine in other reactions. Since B_{12} usually increased the uric acid level of the blood, perhaps this vitamin functions in the metabolic pathways concerned in the degradation of amino acids and the excretion of amino nitrogen as uric acid. If this were so, an excess of amino nitrogen could be better tolerated in B_{12}-replete animals, due to its more rapid catabolism and excretion as uric acid.

SUMMARY

1. Addition of 3, 6, and 9% glycine to vitamin B_{12}-deficient diets caused a growth depression and increased mortality in growing chickens.

2. Both vitamin B_{12} and folic acid function in counteracting this toxicity; however, B_{12} is somewhat more effective.

3. Addition of 6 or 9% glycine to the diet increased blood uric acid levels. Folic acid tended to decrease these high levels of uric acid in the blood.

4. Vitamin B_{12} tends to increase the level of uric acid in the blood of young chickens.

LITERATURE CITED

BOSSHARDT, D. K., W. J. PAUL AND R. H. BARNES 1950 The influence of diet composition on vitamin B_{12} activity in mice. J. Nutrition, *40:* 595.

BRIGGS, G. M. 1946 Nicotinic acid deficiency in turkey poults and the occurrence of perosis. Ibid., *31:* 79.

BROWN, H. 1945 The determination of uric acid in human blood. J. Biol. Chem., *158:* 601.

BUCHANAN, J. M., J. C. SONNE AND A. M. DELLUVA 1948 Biological precursors of uric acid. II. The role of lactate, glycine, and carbon dioxide as precursors of the carbon chain and nitrogen atom 7 of uric acid. Ibid., *173:* 81.

CARY, C. A., A. M. HARTMAN, L. P. DRYDEN AND G. D. LIKELY 1946 An unidentified factor essential for rat growth. Fed. Proc., *5:* 128.

CHARKEY, L. W., H. S. WILGUS, A. R. PATTON AND F. X. GASSNER 1950 Vitamin B_{12} in amino acid metabolism. Proc. Soc. Exp. Biol. Med., *73:* 21.

DINNING, J. S., C. K. KEITH, P. L. DAY AND J. R. TOTTER 1949 PGA, ascorbic acid, and injectable liver extract on dietary glycine toxicity in the rat. Ibid., *72:* 262.

GROSCHKE, A. C., J. O. ANDERSON AND G. M. BRIGGS 1948 Peculiar enlargement of eyeballs in chicks caused by feeding a high level of glycine. Ibid., *69:* 488.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 A role of vitamin B_{12} in the normal mammal. Arch. Biochem., *23:* 165.

KARLSSON, J. L., AND H. A. BARKER 1949 Biosynthesis of uric acid labeled with radioactive carbon. J. Biol. Chem., *177:* 597.

KELLY, B., J. P. TOTTER AND P. L. DAY 1950 The lipotropic effect of folic acid on rats receiving various purified diets. Ibid., *187:* 529.

LIENER, I. E., AND M. O. SCHULTZE 1950 Liver arginase activity as related to blood urea in acute uremia of new-born rats. Ibid., *187:* 743.

LLOYD, M. D., C. A. REED AND J. C. FRITZ 1949 Experiences with high protein diets for chicks and poults. Poultry Sci., *28:* 69.

MCGINNIS, J., P. T. HSU AND W. D. GRAHAM 1948 Studies of an unidentified factor required by chicks for growth and protein utilization. Ibid., *27:* 674.

MENGE, H., AND G. F. COMBS 1950 Action of vitamin B_{12} in counteracting glycine toxicity. Proc. Soc. Exp. Biol. Med., *75:* 139.

MENGE, H., G. F. COMBS, P. T. HSU AND M. S. SHORB 1951 Unidentified factors required by chicks and poults. 1. Studies with chicks using purified diets. Poultry Sci., in press.

PAGÉ, E., AND R. GINGRAS 1949a L'action bacteriostatique du glycocolle et les besoins du rat pyridoxine. Mémoires Soc. Roy. Canada. Troisieme Seire, Sec. V, XLIII.

PAGÉ, E., F. MARTEL AND R. GINGRAS 1949b Changements biochimiques au cours de l'intoxication par le glycocolle 1. Effets de l'acid folique. Rev. Canadienne de Biol., *8:* 298.

RUBIN, M., AND H. R. BIRD 1947 A chick growth factor. V. Relation to quantity and quality of soybean oil meal in the diet. J. Nutrition, *34:* 233.

STERN, J. R., AND J. MCGINNIS 1951 Toxicity of glycine for vitamin B_{12} deficient chicks. Proc. Soc. Exp. Biol. Med., *76:* 233.

ZUCKER, L. M., AND T. F. ZUCKER 1948 Zoopherin: A nutritional factor required by chicks for growth and protein utilization. Arch. Biochem., *16:* 115.

THE DOMESTIC CAT AS A LABORATORY ANIMAL FOR EXPERIMENTAL NUTRITION STUDIES

III. NIACIN REQUIREMENTS AND TRYPTOPHAN METABOLISM

ALBERTO CARVALHO DA SILVA, RAINER FRIED AND REBECCA CARLOTTA DE ANGELIS[1]

Department of Physiology,[2] Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil

ONE FIGURE

(Received for publication November 8, 1951)

In recent years it has been shown that the niacin requirements of many animal species can be satisfied by the administration of tryptophan, and that this amino acid increases the formation of niacin metabolites. This was first established for the rat by Krahl et al. ('45b) and later was found by numerous investigators to be true also for the mouse, chick, guinea pig, calf and cow, horse, pig, rabbit, and man. In contrast to this finding for the several animal species just listed, with the exception of *Drosophila* (Schultz and Rudkin, '48) and other insects (Fraenkel and Stern, '51), we have found that in the domestic cat tryptophan cannot replace niacin, at least on a 35% casein diet, whether the amino acid is given orally or by parenteral administration. This has been demonstrated by studies of growth as well as by determination of niacin metabolites. It may be concluded that in the cat the metabolism of tryptophan is different from that in other species. This paper is a report of experiments supporting this conclusion.

EXPERIMENTAL

The detailed techniques used in working with cats have already been described (Carvalho da Silva, '50a). The animals

[1] On a grant from the Merck Institute for Therapeutic Research.

[2] Head: Professor Franklin A. de Moura Campos.

were housed separately in screen-bottom cages kept in a constant-temperature room. The cats were received from dealers when from 8 to 10 weeks of age. Each animal was given vermifuge and then maintained on stock ration until well adapted to the laboratory. When "adult" cats are mentioned in this report, we refer either to animals brought in from outside or taken from other experiments in our colony.

Diets used

The *stock ration* had the following percentage composition: crude casein, 10; slightly cooked and compressed oats, 20; cooked and mashed potatoes, 15; raw beef muscle, 20; sardines, eviscerated and steamed, 20; lard, 10; bone meal, 2; and cod liver oil, 3. The percentage composition of the *purified ration* was as follows: "vitamin-free" casein,[3] 35; gelatin, 1; sucrose, 48; peanut oil, 5; lard, 5; salt mixture IV (Phillips and Hart, '35), 5; "ruffex,"[4] 1. Both rations were mixed with water and supplied as a paste. The *purified diet* was supplemented with vitamins administered separately. One milligram each of thiamine, riboflavin, pyridoxine, vitamin K and para-aminobenzoic acid; 4 mg of calcium pantothenate; 0.5 mg of folic acid; 0.05 mg of *d*-biotin; 30 mg of inositol; and 300 mg of choline were dissolved in 1 ml of water, this mixture emulsified in 2 ml of cod liver oil, and the resulting product given by mouth to each animal three times a week; once a week, 15 mg of alpha-tocopherol were added. Repeated trials in previous experiments (Carvalho da Silva, '50b) proved that ascorbic acid is not required. It has been demonstrated that this purified ration, when supplemented with 4 mg of niacin three times a week, promotes a satisfactory growth rate and blood picture and maintains the animal in good health for at least 12 months (Carvalho da Silva, '50b).

When tryptophan was given orally, the appropriate amount was emulsified with 2 ml of peanut oil and given with a syringe

[3] "Labco" brand, obtained from the Borden Company, New York. N. Y.
[4] From the Fischer Scientific Company.

without a needle. For subcutaneous administration, it was mixed with 3 or 4 ml of saline directly in a lubricated syringe and rapidly injected with a large-bore needle, attention being paid to asepsis. Injected niacin or nicotinamide was dissolved in 1 ml of physiological saline.

Urine was collected during 48 hours and the samples pooled. Feces were also collected for a 48-hour period when the animals defecated daily; otherwise, two successive samples were collected and the results divided by the total number of days. To separate feces from urine the animal was placed in a screen-bottom cage 12 × 25 inches in size; under the cage was placed an enameled tray covered with a fine wet cloth supported by a wire screen. Diarrhea, a common occurrence during niacin deficiency, made it very difficult properly to separate the feces from urine. All samples were kept below + 5°C. until analyzed.

N^1-methylnicotinamide was determined fluorometrically (Huff and Perlzweig, '47). Microbioassay by *L. arabinosus* was used for the determination of niacin (Krehl et al., '45a) and tryptophan (Greene and Black, '44). For the tryptophan analyses urines were assayed without previous treatment; feces, however, were autoclaved with barium hydroxide for 7 hours at 15 pounds pressure and then, after careful precipitation of the barium, extracted with ether and toluene to eliminate anthranilic acid and indol.

RESULTS

Young cats, when fed the purified ration without niacin, stopped growing after from 10 to 15 days; they then lost weight, and died in from 15 to 50 days; adult animals lost weight and eventually died. The sex of the animal was without influence. Frequently cats that were two or three months old when placed on this dietary regime lost weight from the very beginning. During the deficiency period they frequently showed unkempt fur and diarrhea but no skin or buccal lesions. Blood cell counts failed to demonstrate the presence of anemia. Association with respiratory diseases seemed to be very fre-

quent and to contribute to early death, at least in young animals.

This general pattern of response to the diet was observed in 45 cats. We have no record of a single cat being able to grow for more than 20 days on a niacin-deficient ration. All of the 21 animals which failed to receive niacin died.

The administration of niacin or nicotinamide was usually followed in 24 to 48 hours by a sharp gain in body weight of 50 or more gm per day (fig. 1). Such therapy was sometimes ineffective, however, particularly if the animals had already contracted a serious respiratory infection. The minimum effective dose given subcutaneously proved to be 1 to 3 mg. This may promote a total weight-gain response of 100 to 200 gm during a period of 4 to 8 days; frequently the response is not more than an interruption in weight loss. If one may judge by the period of growth occurring after a single dose, the body's capacity for storage of the vitamin is small. The optimum dose appears to be in the neighborhood of 10 mg.

Data on the amount of niacin in the liver are given in table 1. It will be noticed that the livers of cats on the stock ration had more niacin than those of animals on the purified diet, whether or not it was supplemented by niacin.

Ellinger and Kader ('49) reported that the cat can methylate nicotinamide. Our data confirm this (table 2). However, when tryptophan was administered, either orally or subcutaneously, there was no growth response (fig. 1) and there was no regular increase in nicotinamide excretion (table 2). It can be seen from table 3 that tryptophan was retained in the organism and not excreted in the feces or urine.

A few tests were carried out in an attempt to identify a possible step in tryptophan transformation. It has been demonstrated (Sarett and Goldsmith, '49) that in human pellagra D-tryptophan can be partially excreted in the urine as an ether-soluble substance that shows tryptophan activity for *L. arabinosus*. This substance may be removed by ether extraction and the residual activity considered as representing tryptophan. Judged by the *L. arabinosus* assay, small quantities

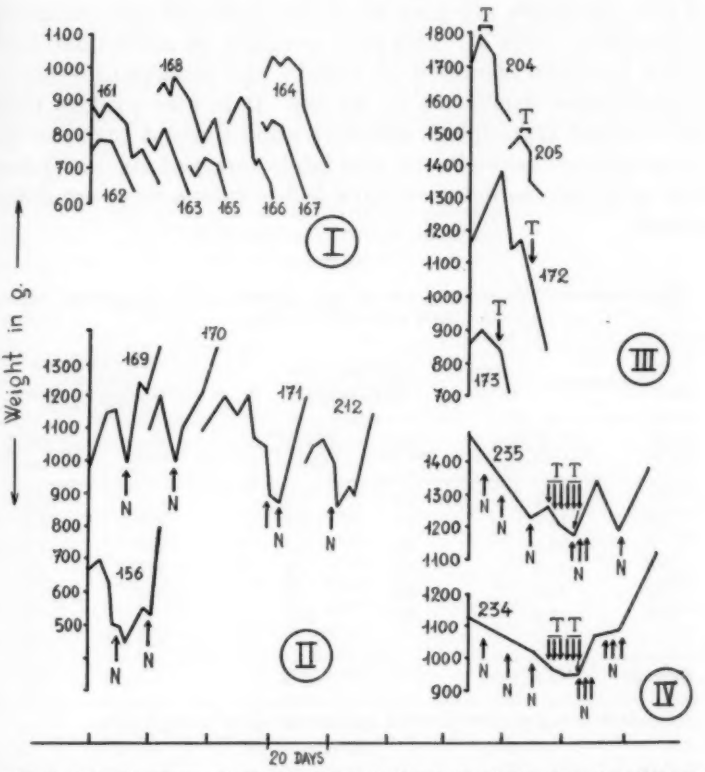

Fig. 1 Growth curves of cats fed diets deficient in niacin, and the effect of supplementation with niacin and tryptophan. The number on each curve corresponds to our laboratory number of the animal. I: Curves of rats not receiving niacin. The end of the curve corresponds to the death of the animal. II: N signifies that niacin was given in amounts of from 10 to 25 mg. III: T indicates that tryptophan was given, twice in doses of 50 mg orally (204 and 205) and once as 50 mg subcutaneously (172 and 173). The end of the curve corresponds to the death of the animal. IV: The animals were maintained for about a month on a small niacin intake (1 to 3 mg) as indicated by N. The object here was to maintain them on the niacin deficiency regime for a long period. At the end of this period the cats received 400 mg tryptophan subcutaneously daily for three days and 600 mg orally for each of three days. When tryptophan supplementation was terminated, niacin in doses of 3 mg was given in order to prove that the "sickness" of the animals was due to niacin deficiency and not to something else. The tryptophan was always the DL form. With groups II and IV, all of the cats shown here attained much greater body weights; the curves are interrupted in order to save space.

of this substance are present in the urines of cats receiving tryptophan (table 3). This may, perhaps, be anthranilic acid, which has been identified by Kotake and Nakayama ('41) as a tryptophan derivative in the cat. It is also evident from table 3 that tryptophan administration did not increase the formation of free or bound acid-labile forms of niacin. Quinolinic acid and methyl-nicotinamide-6-pyridone were not determined.

TABLE 1

Niacin content of the livers of cats on stock rations, and on the purified ration with and without niacin

STOCK RATION		PURIFIED RATION WITH ALL VITAMIN SUPPLEMENTS		PURIFIED RATION WITH ALL VITAMIN SUPPLEMENTS EXCEPT NIACIN[1]	
Cat number	mg niacin per 100 gm of liver	Cat number	mg niacin per 100 gm of liver	Cat number	mg niacin per 100 gm of liver
42	12.4	10	7.3	156	10.6
43	14.6	11	8.3	172	9.8[1]
82	15.3	35	11.1	173	4.2[1]
200	11.8	64	9.8	210	9.6
202	12.6	85	6.4	221	13.2
204	13.9	90	14.3	222	7.2
				223	11.5
Average	13.4		9.7		9.4

[1] Received 50 mg of DL-tryptophan subcutaneously as a supplement.

Other evidence suggests a special tryptophan metabolism in the cat. Four animals, two months old, were fed the purified ration supplemented by all vitamins except pyridoxine. When they were deficient of pyridoxine, as judged by the presence of anemia and interruption of growth, they received two oral supplements of 100 mg of DL-tryptophan given on successive days. No xanthurenic acid was found in the urine by a qualitative test with iron salts (Lepkovsky et al., '43).

DISCUSSION

Heath et al. ('40) reported a regression of deficiency symptoms in cats on diets of natural foods by treatment with nico-

tinamide; unfortunately their brief report gives no experimental details. In view of the results obtained with other species, the inability of the cat to utilize tryptophan for niacin replacement is quite surprising. Although it does not seem very likely, it is possible that higher levels of casein would be

TABLE 2

The effect of nicotinamide (N) and DL*-tryptophan (T) on urinary excretion of N^1-methylnicotinamide*

CAT NUMBER	RATION	SUPPLEMENT			N^1-METHYLNICOTINAMIDE	
					Before receiving supplement	After receiving supplement
					μg/day	*μg/day*
216	Stock	N	5	subcut.	700	3,000
217	Stock	N	5	subcut.	800	1,600
218	Stock	N	15	subcut.	800	2,900
219	Stock	N	20	subcut.	900	5,900
220	Stock	N	30	subcut.	400	8,300
169	Purified [1]	N	50	subcut.	0	14,400
172	Purified	N	50	subcut.	200	15,500
171	Purified	N	100	subcut.	250	41,700
207	Purified	N	200	subcut.	200	26,000
216	Stock	T	40	orally [2]	550	350
217	Stock	T	40	orally [2]	350	380
218	Stock	T	40	orally [2]	450	550
219	Stock	T	40	orally [2]	550	500
220	Stock	T	40	orally [2]	550	400
170	Purified [1]	T	50	subcut.	140	200
172	Purified	T	50	subcut.	200	300
173	Purified	T	50	subcut.	150	0
221	Purified	T	100	subcut. [2]	200	300
224	Purified	T	100	subcut. [2]	300	150
234	Purified	T	400	subcut. [2]	200	550
235	Purified	T	400	subcut. [2]	100	300
236	Purified	T	400	subcut. [2]	200	200
234	Purified	T	600	orally [2]	(200) [3]	150
235	Purified	T	600	orally [2]	(200) [3]	100
236	Purified	T	600	orally [2]	(200) [3]	350

[1] All of the animals on the purified ration were niacin-deficient.

[2] This amount was given daily for three successive days.

[3] With cats 234, 235 and 236 no determination was made for N^1-methylnicotinamide before oral supplementation with tryptophan. The values obtained before the subcutaneous supplementation are tabulated in parentheses.

TABLE 3

Influence of tryptophan supplements on tryptophan and niacin excretion by cats on purified ration

CAT NUMBER	TRYPTOPHAN SUPPLEMENT	URINE				FECES	
		Tryptophan content		Niacin content		Tryptophan content	Niacin content
		Extracted [1]	Not extracted	Autoclaved	Not Autoclaved	Extracted	Autoclaved
	mg	*mg/day*	*mg/day*	*μg/day*	*μg/day*	*mg/day*	*μg/day*
216	0	0.212	0.170	10	..	6.56	56
221	0	0.366	0.441	56	52	..	..
223	0	0.550	0.950	34	37	7.45	35
224	0	0.143	0.266	50	44	7.50	20
224	0	0.400	0.716	74	56	..	..
234	0	0.109	0.131	23	21	11.50	750
235	0	0.163	0.156	35	36	18.80	750
236	0	0.249	0.366	65	45	5.96	995
219	50 (L) orally	0.165	0.186	21	88	8.8	134
220	50 (L) orally	0.310	0.430	58	..	8.7	88
222	50 (L) orally	0.145	0.232	21	25	7.6	48
217	100 (DL) orally	0.225	0.325	38	..	7.0	..
218	100 (DL) orally	0.233	0.300	20	..	8.8	130
221	100 (DL) orally	0.110	0.110	47	72	7.9	95
234	600 (DL) orally [2]	0.460	0.500	21	21	22.4	500
235	600 (DL) orally [2]	0.420	0.460	15	21	18.8	775
236	600 (DL) orally [2]	0.833	1.125	33	24	50.4	885
221	100 (DL) subcut.[2]	0.176	0.151	41	32	..	..
224	100 (DL) subcut.[2]	0.225	0.195	50	46	..	..
234	400 (DL) subcut.[2]	0.115	0.146	26	17	5.0	345
235	400 (DL) subcut.[2]	0.112	0.112	21	19	14.5	1,050
236	400 (DL) subcut.[2]	0.310	0.342	58	70	8.2	480

[1] Ether and toluene extract according to Sarett and Goldsmith ('49); see text.

[2] This amount given daily for three successive days.

protective. This might perhaps be considered as ruled out by the fact that supplying additional tryptophan did not promote growth. Still, it does seem remarkable that a diet with 35% casein should be without effect, when in the rat a ration containing only 8% casein supplies sufficient tryptophan to satisfy the niacin requirement of the animal.

In view of the spontaneous variation in the excretion of N^1-methylnicotinamide even by niacin-deficient cats, it cannot be stated on the basis of the data presented here that there is no conversion of tryptophan to niacin in the cat. It is evident, however, that if such conversion does occur, it is in degree much smaller than in other mammals and is insufficient to meet the niacin requirements, at least on the levels tried. If the niacin requirement in this species is high, the observations would be explained. On the other hand, high niacin requirements could be equally well explained by a reduced or non-existent capacity to obtain niacin from tryptophan.

Our results confirm the hypothesis of Jackson ('29) that tryptophan metabolism of cats is different from that in other mammals. In a series of experiments he verified that no kynurenic acid can be isolated from the urine of cats kept on a high protein diet even when the animals receive supplemental tryptophan. In our studies two other major metabolites of tryptophan, namely xanthurenic acid and nicotinic acid, could not be obtained from the urine of cats.

Kotake and Nakayama ('41) have shown that in the cat tryptophan is degraded to anthranilic acid. This was confirmed by Braunstein and Goryachenkova ('49), who identified pyridoxal phosphate as the coenzyme involved in the reaction. These results, considered together with those of Jackson as well as our own, indicate that the degradation of tryptophan follows the pathway characteristic of other species up to kynurenine, but differs from this stage onward. The complete pathway is as yet unknown. If kynurenine should be quantitatively transformed into anthranilic acid and alanine, as stated by Kotake and his associate, this would explain

This amount given daily for three successive days.

the failure to obtain kynurenic acid, xanthurenic acid and niacin.

The degradation of nicotinamide in the cat follows the generally accepted reaction scheme: after administration of nicotinic acid or nicotinamide, high levels of N^1-methylnicotinamide are excreted in the urine, an observation also made by Ellinger and Kader ('49).

SUMMARY

Niacin deficiency, characterized by loss in body weight, diarrhea and death, has been produced in cats fed a 35% casein diet.

Doses of 1 to 3 mg of niacin are effective against the deficiency; the optimum appears to be 10 mg or more. Tryptophan supplements are not effective either in promoting growth or in bringing about urinary excretion of N^1-methylnicotinamide. Cats deficient in pyridoxine do not excrete xanthurenic acid following supplementation with tryptophan.

It is concluded that in the cat the metabolism of tryptophan is different from that in other species, at least from the kynurenine stage onward.

ACKNOWLEDGMENTS

The authors take this opportunity to thank: (a) the Rockefeller Foundation, for providing the special vitamin-free casein and vitamins needed for these studies; (b) Dr. E. Sevringhaus of Hoffmann La-Roche, Inc., for the *d*-biotin used; and (c) "Commisão da Pesquiza Cientifica da Universidade de S. Paulo" for its assistance.

LITERATURE CITED

BRAUNSTEIN, A. E., AND E. B. GORYACHENKOVA 1949 Enzymatic formation of alanine from L-kynurenine and L-tryptophan and the role of vitamin B_6 in the process. Biekhimia, *14:* 163 (through Nutrition Abstr. and Rev., *19:* 869, 1950).

CARVALHO DA SILVA, A. C. 1950a The domestic cat as a laboratory animal for experimental nutrition studies. I. General considerations, care and feeding of the animals. Acta Physiol. Lat. Amer., *1:* 20.

CARVALHO DA SILVA, A. C. 1950b The domestic cat as a laboratory animal for experimental nutrition studies. II. Comparative growth and hematology on stock and purified rations. Ibid., *1:* 26.

ELLINGER, P., AND M. M. ABDEL KADER 1949 Nicotinamide metabolism in mammals. Biochem. J., *49:* 77.

FRAENKEL, G., AND H. R. STERN 1951 The nicotinic acid requirements of two insect species in relation to the protein content of their diets. Arch. Biochem., *30:* 438.

GREENE, R. D., AND A. BLACK 1944 The microbiological assay of tryptophan in proteins and feeds. J. Biol. Chem., *155:* 1.

HEATH, M. K., J. W. MACQUEEN AND T. D. SPIES 1940 Feline pellgra. Science, *92:* 514.

HUFF, J. W., AND W. A. PERLZWEIG 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. II. A sensitive method for the determination of N^1-methylnicotinamide in urine. J. Biol. Chem., *167:* 157.

JACKSON, R. W. 1929 The excretion of kynurenic acid by members of various families of the order carnivora. Ibid., *131:* 469.

KOTAKE, Y., AND Y. NAKAYAMA 1941 Studien über den intermediären Stoffwechsel des Tryptophans. XXXIV. Über die Anthranilsaurebildung aus Kynurenin durch Organsaft. Zschr. physiol. Chem., *270:* 76.

KREHL, W. A., F. M. STRONG AND C. A. ELVEHJEM 1945a Determination of nicotinic acid: Modifications of the microbiological method. Ind. and Eng. Chem., Anal. Ed., *15:* 471.

KREHL, W. A., L. J. TEPLEY AND C. A. ELVEHJEM 1945b Corn as an etiological factor in the production of nicotinic acid deficiency in the rat. Science, *101:* 283.

LEPKOVSKY, S., E. ROBOZ AND A. J. HAAGEN-SMIT 1943 Xanthurenic acid and its role in the tryptophan metabolism of pyridoxine-deficient rats. J. Biol. Chem., *149:* 195.

PHILLIPS, R. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. Ibid., *109:* 657.

SARETT, H. P., AND G. A. GOLDSMITH 1949 Tryptophan and nicotinic acid studies in man. Ibid., *177:* 461.

SCHULTZ, J., AND G. T. RUDKIN 1948 Absence of a sparing action of tryptophan on nicotinamide requirements of the fly *Drosophila melanogaster*. Fed. Proc., *7:* 185.

THE EFFECT OF DIFFERENT CARBOHYDRATES AND ANTIBIOTICS ON THE GROWTH OF CHICKS AND THE STORAGE OF VITAMINS [1]

W. J. MONSON, L. S. DIETRICH AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication December 5, 1951)

Previous work showed that when the rate of growth of chicks was used to measure the nutritional efficiency of carbohydrates they fell in the following order: dextrin, cerelose, sucrose, and lactose (Monson, Dietrich and Elvehjem, '50). The addition of known vitamins did not alter the responses obtained with the different carbohydrates. More recently Dietrich, Monson and Elvehjem ('51a) reported that the addition of certain natural materials to rations containing sucrose produced a growth response similar to that observed with dextrin as the carbohydrate.

It has been noted by several workers that the type of carbohydrate influences the level of certain vitamins in the tissues

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by funds supplied by the Commercial Solvents Corporation, Terre Haute, Ind., by Swift and Co., Chicago, Ill., and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

We are indebted to Merck and Co., Rahway, N. J., for some of the crystalline vitamins, crystalline penicillin, and the streptomycin concentrate; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid; to Commercial Solvents Corporation, Terre Haute, Ind., for bacitracin; to Wilson and Co., Inc., Chicago, Ill., for gelatin; to Abbott Laboratories, North Chicago, Ill., for haliver oil; and to E. I. DuPont de Nemours and Co., Inc., New Brunswick, N. J., for crystalline vitamin D_3.

of animals and the eggs of hens. Much of this work has been reviewed by Elvehjem ('46, '48).

The work reported here was undertaken to determine if the differences in growth obtained with different carbohydrates could be correlated with possible changes in the vitamin content of the livers and ceca taken from individual chicks. The chicks were fed rations supposedly adequate in all the known vitamins, since the growth differences were obtained under such conditions. Studies were also made with antibiotics to determine if the growth responses obtained with these supplements could be correlated in any way with vitamin storage. Again the antibiotics were added in the presence of adequate levels of known vitamins, and the results should not be compared with those of Linkswiler et al. ('51) and Lih and Baumann ('51) with the rat or Biely and March ('51) with the chick, which were obtained when certain vitamins were limiting.

EXPERIMENTAL

Straight run (New Hampshire ♂ ♂ × single comb White Leghorns ♀ ♀) cross-bred chicks, which were the progeny of hens fed diet B-1 described previously, were used in all studies (Robblee and co-workers, '48). The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied ad libitum. The chicks were wing-banded and weighed at one day of age. Weights were recorded at weekly intervals.

All chicks were divided into groups according to weight and immediately placed on test for 4 weeks. They all received a semi-purified ration containing carbohydrate 61 gm, alcohol-extracted casein 18 gm, gelatin 10 gm, Salts V (Briggs et al., '43) 6 gm, soybean oil 5 gm, L-cystine 0.3 gm, thiamine hydrochloride 0.3 mg, riboflavin 0.6 mg, nicotinic acid 5.0 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2.0 mg, choline chloride 150 mg, biotin 0.03 mg, inositol 100 mg, folic acid 0.2 mg, vitamin B_{12} 0.002 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, and α-tocopherol 0.3 mg. Fortified haliver oil (60,000

U.S.P. units of vitamin A, 6,000 U.S.P. units of vitamin D_3 per gram) was given by dropper (two drops per bird per week). The antibiotics, when used, were added to the complete ration. At the end of the 4-week test period the birds were sacrificed and the livers removed and stored at freezing temperatures until they were prepared for assay.

The cecal samples were prepared by homogenizing a weighed amount of the cecal contents in a Potter-Elvehjem homogenizer and aliquots were taken for dry weight determination and vitamin extraction. Folic acid, vitamin B_{12} and *Leuconostoc citrovorum* factor [3] extractions were made in pH 7 citrate-phosphate buffer and vitamin B_6 extraction was made in 0.055 N HCl. The samples were then diluted to volume, filtered and assayed as indicated below. Liver folic acid and vitamin B_{12} were extracted and assayed as described previously (Dietrich et al., '49).

An aliquot was taken from the samples prepared for the folic acid assay and used in the assay for the *Leuconostoc citrovorum* factor (C.F.) by the method of Sauberlich and Baumann ('48). This has been shown to give maximum C.F. activity (Dietrich, Monson, Gwoh and Elvehjem, '51b).

Pantothenic acid was extracted by adding 2.5 ml of the homogenate previously described containing 0.5 gm liver (Dietrich et al., '49) to 25 ml H_2O and autoclaving for 15 minutes at 120°C. The preparation was cooled, 50 ml of citrate-phosphate buffer at pH 4.5 and 50 mg of takadiastase were added, and the flasks were incubated under toluene for 18 hours. The samples were then autoclaved for 5 minutes at 120°C., cooled, diluted, filtered, and assayed by the method of Skeggs and Wright ('44) using *L. arabinosus* as the test organism.

Biotin was extracted by autoclaving 2.5 ml of the homogenate in 6 N HCl for two hours at 120°C. The samples were cooled, neutralized, diluted, filtered, and assayed by the method

[3] We are deeply indebted to Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for the crystalline calcium salt of *Leuconostoc citrovorum* factor.

of Wright and Skeggs ('44) using *L. arabinosus* as the test organism.

Niacin was extracted by autoclaving 2.5 ml of the homogenate in 25 ml of 1 *N* HCl for 15 minutes at 120°C. The samples were cooled, neutralized, diluted, filtered and assayed, using the media resulting from a collaborative U. S. Pharmacopeia and American Association of Agricultural Chemists study ('45) and *L. arabinosus* as the test organism.

Vitamin B_6 was extracted by autoclaving 2.5 ml of the homogenate in 150 ml of 0.055 N HCl for 5 hours at 120°C. The samples were cooled, diluted, filtered, and assayed by the method of Atkin et al. ('43) using *S. carlsbergensis* 4228 as the test organism.

Aliquots from the samples prepared for the biotin assay were used for the assay of inositol. The method of Atkin et al. ('44) was employed, using *S. carlsbergensis* 4228 as the test organism.

Total choline was determined by a Reineckate method (Biological Symposia, '47).

RESULTS AND DISCUSSION

The 4 experiments made to determine the effect of various carbohydrates on the storage of several water-soluble vitamins in chick liver are summarized in table 1. Although some of the growth data have been reported by Monson et al. ('50), the records are included here to facilitate correlation with the vitamin levels.

The levels of vitamin B_{12}, vitamin B_6, pantothenic acid, and niacin were similar regardless of the type of carbohydrate fed. The high pantothenic acid values in the dextrin and lactose groups obtained in experiment 2 were not obtained in experiment 4. The inconsistencies found may have been due to the inoculum received by the chick in the first food and not necessarily to the type of carbohydrate.

There was a trend toward higher biotin storage in the dextrin-fed chicks. This was especially true in experiment 2. However, in experiment 4 the standard errors for all groups

overlapped and the significance is questionable. It should be noted that Couch et al. ('48) found more biotin in the eggs from hens fed a ration containing dextrin as the carbohydrate than in the eggs of hens fed a sucrose ration.

The chicks fed a lactose-containing diet appeared to have less inositol and choline in their livers than did the chicks fed sucrose or dextrin. Whether this was the cause of the inferior growth of the chicks receiving lactose is not certain, since there are other physical abnormalities, such as excessive diarrhea, that must be considered.

The most clear-cut differences noted were the high folic acid and C.F. contents of the livers of chicks fed lactose. A reasonable explanation is the favorable influence that lactose may have on the folic acid- and C.F.-synthesizing flora in the chick intestine. Teply et al. ('47) found that the addition of lactose to the basal ration of rats increased the synthesis of both niacin and folic acid. Bacigalupo et al. ('50) have shown that cotton rats have more intestinal synthesis of folic acid when fed lactose than when fed sucrose. It is of interest that despite the extremely high level of these very important factors, the chicks fed lactose grew poorly. The poor growth probably was due either to diarrhea or to a limiting supply of some nutrient other than those mentioned above. The levels of folic acid in the livers of chicks fed sucrose, dextrin, or corn starch were very similar.

There appeared to be a trend toward higher C.F. in the livers of chicks fed a dextrin ration than in the livers of those receiving sucrose. The dextrin may indirectly cause more efficient conversion of folic acid to C.F. However, this does not explain why the chicks in the sucrose and cornstarch groups should have had the same amount of C.F. in their livers.

The cecal concentrations of a few of the vitamins were determined for chicks in experiment 4 (table 2). The level of folic acid was higher in the ceca of chicks fed cerelose than in those of chicks receiving sucrose or dextrin. It should be noted that the liver levels were the same for these groups, so

TABLE 1

Effect of various carbohydrates on the storage of several water-soluble vitamins in chick liver [1]

TYPE OF CARBOHYDRATE IN DIET		SUCROSE	DEXTRIN	LACTOSE	CERELOSE	CORN STARCH
		gm	gm	gm	gm	gm
4-Week weight gain	(1)	193	252	142	...	...
	(2)	196	280	149	...	...
	(3)	222	305	155	259	...
	(4)	190	260	...	247	240
Vitamin levels		µg/gm	µg/gm	µg/gm	µg/gm	µg/gm
Vitamin B_{12}	(1)	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	...	...
	(2)	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	...	...
	(3)	0.06 ± 0.00	0.08 ± 0.00	0.08 ± 0.01	0.09 ± 0.00	...
	(4)	0.04 ± 0.00	0.05 ± 0.00	...	0.06 ± 0.00	0.05 ± 0.00
Leuconostoc citrovorum factor [2]	(1)	...	...	...	...	...
	(2)	2.28 ± 0.18	4.00 ± 0.26	14.69 ± 2.34	...	...
	(3)	3.03 ± 0.17	4.04 ± 0.26	47.50 ± 3.30	8.71 ± 0.50	...
	(4)	6.4 ± 0.55	...	...	4.5 ± 0.67	6.5 ± 1.24
Folic acid	(1)	2.36 ± 0.18	1.74 ± 0.20	17.63 ± 2.32	...	...
	(2)	3.55 ± 0.43	3.36 ± 0.49	40.55 ± 1.58	...	...
	(3)	3.11 ± 0.13	2.69 ± 0.53	32.0 ± 1.76	6.0 ± 0.67	...
	(4)	2.59 ± 0.16	2.66 ± 0.24	...	2.20 ± 0.2	2.78 ± 0.45
Vitamin B_6	(1)	...	...	...	...	...
	(2)	...	...	...	...	...
	(3)	...	...	...	...	...
	(4)	9.0 ± 0.49	12.0 ± 0.17	...	11.0 ± 0.42	12.4 ± 0.43
Pantothenic acid	(1)	...	...	...	...	...

Pantothenic acid	(1)	...	...	...	...	...
	(2)	20.00 ± 4.47	48.00 ± 9.1	46.00 ± 3.28	...	...
	(3)	...	...	...	...	...
	(4)	21.2 ± 1.81	22.5 ± 0.73	...	25.7 ± 1.69	32.6 ± 4.16
Niacin	(1)	...	...	...	...	...
	(2)	96.0 ± 6.4	97.0 ± 4.1	100.0 ± 2.05	...	...
	(3)	...	...	...	...	...
	(4)	133 ± 23.3	132 ± 22.7	...	126 ± 6.0	139 ± 4.23
Biotin	(1)	...	...	...	...	...
	(2)	2.74 ± 0.40	4.00 ± 0.59	2.69 ± 0.70	...	...
	(3)	...	...	...	...	...
	(4)	2.53 ± 0.51	3.16 ± 0.32	...	3.62 ± 0.21	3.66 ± 0.47
Inositol	(1)	...	...	...	...	...
	(2)	7,400 ± 300	7,700 ± 563	3,600 ± 329	...	...
	(3)	...	...	...	...	...
	(4)	...	...	...	...	...
Choline	(1)	...	...	...	...	...
	(2)	1,028 ± 343	1,200 ± 222	552 ± 188	...	...
	(3)	...	...	...	...	...
	(4)	...	...	...	...	...

[1] Five observations/group including standard error, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[2] Converted from Reticulogen units (1 mμg C.F. = 0.16 μl Reticulogen).

that the activity here measured is either not absorbed or is not stored. There was a similar situation with respect to vitamin B_{12}. The chicks fed sucrose had a higher cecal concentration of vitamin B_{12} than chicks fed the other diets, but the liver levels of all these groups were the same.

The cecal contents were asayed for C.F. activity but no activity was found with the concentrations used. Unpublished data from this laboratory have shown that there is a significant amount of C.F. in rat and guinea pig intestines. Therefore it is possible that some activity can be found in the chick ceca with more concentrated samples. Vitamin B_6 was low

TABLE 2

Effect of various carbohydrates on the cecal concentrations of various water-soluble vitamins[1]

TYPE OF CARBOHYDRATE IN RATION	FOLIC ACID, DRIED WT.	VITAMIN B_{12}, DRIED WT.	VITAMIN B_6, DRIED WT.
	μg/gm	*μg/gm*	*μg/gm*
Sucose basal	10.99 ± 1.96	5.34 ± 1.11	34.5 ± 4.9
Dextrin basal	8.44 ± 0.88	2.74 ± 0.45	44.3 ± 9.8
Cerelose basal	20.9 ± 3.2	1.93 ± 0.24	48.4 ± 11.1
Corn starch basal	14.55 ± 2.5	1.64 ± 0.08	18.4 ± 2.6

[1] Five observations/group including standard error, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

in the ceca of chicks fed a cornstarch ration, but again the liver levels of all 4 groups were the same.

The levels of the vitamins tested were essentially the same in the livers of chicks fed dextrin or sucrose as the source of carbohydrate. We have reported (Monson et al., '50) that doubling the level of all water-soluble vitamins in the ration did not change the growth differences noted with the basal ration. These two types of experiments show that the superior growth obtained with dextrin is not due to the production of known vitamins.

Effects of antibiotics

Table 3 shows the effect of supplementing the dextrin basal ration with various antibiotics and sulfasuxidine, singly

and in combinations. Very good growth responses were observed, the best being in the group supplemented with both bacitracin and streptomycin. Although each of the antibiotics produced a growth response when fed alone, bacitracin plus penicillin, and all three fed together, produced no better growth than did the control ration. The addition of sulfa-

TABLE 3

The effect of supplementing the diet with antibiotics on the growth of chicks fed rations containing dextrin or sucrose as the carbohydrate

SUPPLEMENT	LEVEL FED	EXP. 1 (12 BIRDS/GROUP)	EXP. 2 (12 BIRDS/GROUP)	
		Dextrin 4-week wt. gain	Dextrin 4-week wt. gain	Sucrose 4-week wt. gain
		gm	*gm*	*gm*
None		286	298	225
Sulfasuxidine	2%	244	...	...
Streptomycin	100 mg%	314	...	...
Streptomycin	50 mg%	...	315	214
Penicillin	100 mg%	310	...	...
Penicillin	50 mg%	...	317	214
Bacitracin	6 mg%	320	313	248
Bacitracin + penicillin	6 mg% + 100 mg%	272	...	...
Bacitracin + penicillin	6 mg% + 50 mg%	...	315	290
Bacitracin + streptomycin	6 mg% + 100 mg%	353	...	...
Bacitracin + streptomycin	6 mg% + 50 mg%	...	315	251
Bacitracin + streptomycin + penicillin	6 mg% + 100 mg% + 100 mg%	289	...	...
Bacitracin + streptomycin + penicillin + sulfasuxidine	6 mg% + 100 mg% + 100 mg% + 2%	225	...	...

suxidine caused inferior growth when fed alone or in combination with the three antibiotics. It appears that certain combinations of antibiotics exhibit a synergistic effect. It has been reported by Bachman ('49) that bacitracin and penicillin, *in vitro,* have a synergistic effect on the inhibition of various streptococci. It has also been reported that aureomycin and terramycin interfere with the action of penicillin *in vitro* (Gunnison et al., '50). Here we have, first, the action of one antibiotic counteracting the growth-stimulating power of another and, second, the action of one antibiotic enhancing the growth-promoting power of another.

It becomes apparent that certain other factors influence these effects. In experiment 2 the amounts of streptomycin and penicillin added as supplements were cut in half. All three antibiotics fed singly, as well as the two combinations tried, gave the same small growth increase on the dextrin ration. On the sucrose ration, however, streptomycin alone and penicillin alone gave no response over that obtained with the control ration. Bacitracin appears to have some activity alone which is not augmented by the addition of streptomycin, the combination showing the most activity in experiment 1. However, the addition of penicillin to the bacitracin gave a significant growth increase over that obtained with bacitracin alone. Although the results varied somewhat from experiment to experiment, the combination of bacitracin and penicillin consistently produced a growth response on a sucrose ration. These variations indicate that the effectiveness of antibiotics depends on the concentrations fed, the type of carbohydrate in the diet, and the nature of the flora established in the intestinal tract.

To determine the effect of antibiotics on the storage of vitamins, the livers from the birds in experiment 1 were assayed for several of the water-soluble vitamins.

Table 4 shows that the levels of pantothenic acid, vitamin B_6, niacin and vitamin B_{12} were similar for all groups. The levels of folic acid and C.F. were higher in those groups in which the chicks received bacitracin than in the other groups.

TABLE 4

Effect of various antibiotics and combinations of antibiotics on liver storage of various B vitamins [1]

	LEVEL FED	FOLIC ACID	CITROVORUM FACTOR [2]	PANTOTHENIC ACID	VITAMIN B_6	NIACIN	VITAMIN B_{12}
		$\mu g/gm$	$\mu g/gm$	$\mu g/gm$	$\mu g/gm$	$\mu g/gm$	$\mu g/gm$
Dextrin control		1.38 ± 0.46	1.88 ± 0.26	20 ± 1.34	10.1 ± 0.27	125 ± 8.65	0.18 ± 0.01
+ sulfasuxidine	2%	2.98 ± 0.22	2.50 ± 0.34	18 ± 0.77	10.0 ± 0.09	106 ± 4.35	0.22 ± 0.02
Streptomycin	100 mg%	1.82 ± 0.29	1.63 ± 0.42	21 ± 1.18	9.8 ± 0.22	130 ± 7.06	0.24 ± 0.03
Penicillin	100 mg%	2.98 ± 0.33	2.50 ± 0.26	22 ± 1.0	10.5 ± 0.35	140 ± 11.4	0.23 ± 0.02
Bacitracin	6 mg%	4.08 ± 0.71	3.59 ± 0.46	20 ± 0.83	10.2 ± 1.45	116 ± 6.78	0.27 ± 0.02
Bacitracin + penicillin	6 mg% 100 mg%	5.28 ± 0.98	4.38 ± 1.30	20 ± 1.34	12.5 ± 0.29	126 ± 10.8	0.20 ± 0.02
Bacitracin + streptomycin	6 mg% 100 mg%	4.98 ± 0.17	3.28 ± 0.33	18 ± 1.22	12.2 ± 0.40	142 ± 6.62	0.23 ± 0.02
Bacitracin + streptomycin + penicillin	6 mg% 100 mg% 100 mg%	5.28 ± 0.58	4.38 ± 0.45	20 ± 1.18	10.6 ± 0.39	140 ± 4.46	0.22 ± 0.03
Bacitracin + streptomycin + penicillin + sulfasuxidine	6 mg% 100 mg% 100 mg% 2%	7.00 ± 1.16	3.28 ± 0.78	22 ± 0.65	11.8 ± 0.79	124 ± 2.45	0.22 ± 0.01

[1] Five observations per group including standard error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[2] Converted from Reticulogen units (1 mμg C.F. = 0.16 μl Reticulogen.)

However, it is very important to note that the chicks that grew the most and those that grew the least had essentially the same amounts of folic acid and C.F. in their livers. There appeared to be no direct correlation between the liver levels of the vitamins tested and the growth of the corresponding chickens.

SUMMARY

1. Chicks fed rations supposedly adequate in all the known vitamins and containing lactose as the carbohydrate had a high concentration of folic acid and C.F. in their livers as compared to chicks fed other carbohydrates.

2. The type of carbohydrate fed chicks receiving adequate amounts of all the known vitamins does not affect the level of several of the water-soluble vitamins found in the livers or ceca in a way which would explain the superior growth obtained on a dextrin ration.

3. The effect of antibiotics on the growth of chicks receiving adequate amounts of all the known vitamins is variable and the concentrations of several of the water-soluble vitamins found in the chick livers cannot be correlated with the growth responses.

LITERATURE CITED

ATKIN, L., A. S. SCHULTZ, W. L. WILLIAMS AND C. N. FREY 1943 Yeast microbiological methods for determination of vitamins — pyridoxine. Ind. Eng. Chem., Anal. Ed., *15:* 141.

ATKIN, L., W. L. WILLIAMS, A. S. SCHULTZ AND C. N. FREY 1944 Preprint of Unpublished Method. Fleischman Laboratories, New York, N. Y. (Cited in Gyorgy, P., Vitamin Methods, Vol. 1, Academic Press, Inc., New York, N. Y., 1950.)

BACHMAN, M. C. 1949 *In vitro* studies on possible synergistic action between penicillin and bacitracin. J. Clin. Invest., *28:* 864.

BACIGALUPO, F. A., J. R. COUCH AND P. B. PEARSON 1950 Effect of sucrose, lactose, galactose, and fructose on fecal excretion of biotin, riboflavin, and PGA in the mature cotton rat. Am. J. Physiol., *162:* 131.

BIELY, JACOB, AND B. MARCH 1951 The effect of aureomycin and vitamins on the growth rate of chicks. Science, *114:* 330.

Biological Symposia 1947 *XII:* 362.

BRIGGS, G. M., JR., T. D. LUCKEY, C. A. ELVEHJEM AND E. B. HART 1943 Studies on two chemically unidentified water-soluble vitamins necessary for the chick. J. Biol. Chem., *148:* 163.

Collaborative study of nicotinic acid assay methods conducted for the American Association of Agricultural Chemists (F. M. Strong, associate referee) and the U. S. Pharmacopeia (O. L. Cline, referee), 1945.

COUCH, J. R., W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN 1948 Relation of carbohydrate to intestinal synthesis of biotin and hatchability in mature fowl. J. Nutrition, *35:* 57.

DIETRICH, L. S., W. J. MONSON AND C. A. ELVEHJEM 1951a Studies on an unidentified growth factor for chicks and hyperthyroid rats fed purified rations. Arch. Biochem., in press.

DIETRICH, L. S., W. J. MONSON, HELEN GWOH AND C. A. ELVEHJEM 1951b Determination of folic acid and citrovorum factor in animal tissue. J. Biol. Chem., *149:* 549.

DIETRICH, L. S., C. A. NICHOL, W. J. MONSON AND C. A. ELVEHJEM 1949 Observations on the interrelation of vitamin B_{12}, folic acid, and vitamin C in the chick. Ibid., *181:* 915.

ELVEHJEM, C. A. 1946 The role of intestinal bacteria in nutrition. J. Am. Dietet. Assn., *22:* 959.

———— 1948 Nutritional significance of the intestinal flora. Fed. Proc., *7:* 410.

GUNNISON, J. G., V. R. COLEMAN AND E. JAWETZ 1950 Interference of aureomycin and of terramycin with action of penicillin *in vitro*. Proc. Soc. Exp. Biol. Med., *75:* 549.

LIH, HWA, AND C. A. BAUMANN 1951 Effects of certain antibiotics on the growth of rats fed diets limiting in thiamine, riboflavin, or pantothenic acid. J. Nutrition, *45:* 143.

LINKSWILER, H., C. A. BAUMANN AND E. E. SNELL 1951 Effect of aureomycin on the response of rats to various forms of vitamin B_6. Ibid., *43:* 565.

MONSON, W. J., L. S. DIETRICH AND C. A. ELVEHJEM 1950 Studies on the effect of different carbohydrates on chick growth. Proc. Soc. Exp. Biol. Med., *75:* 256.

ROBBLEE, A. R., C. A. NICHOL, W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN 1948 The effect of hen diet and chick diet on the need of chicks for an unidentified growth factor. Poultry Sci., *27:* 443.

SAUBERLICH, H. E., AND C. A. BAUMANN 1948 A factor required for the growth of *Leuconostoc citrovorum*. J. Biol. Chem., *176:* 165.

SKEGGS, H. R., AND L. D. WRIGHT 1944 The use of *Lactobacillus arabinosus* in the microbiological determination of pantothenic acid. Ibid., *156:* 21.

TEPLY, L. J., W. A. KREHL AND C. A. ELVEHJEM 1947 Intestinal synthesis of niacin and folic acid in the rat. Am. J. Physiol., *148:* 91.

WRIGHT, L. D., AND H. R. SKEGGS 1944 Determination of biotin with *Lactobacillus arabinosus*. Proc. Soc. Exp. Biol. Med., *56:* 95.

CARIOGENICITY OF SUGAR-CONTAINING DIETS[1]

JOHN HALDI AND WINFREY WYNN
Department of Physiology, Emory University Dental School, Emory University, Georgia

(Received for publication November 9, 1951)

Recent studies by Sognnaes ('48) indicate that caries susceptibility in the Long-Evans strain of the Norway rat may be markedly influenced by systemic factors operating during the period of tooth development. These results have been corroborated by McClure ('51) using the National Institute of Health strain of rats.

The prenatal influence of diet on caries susceptibility, however, appears from the work of these investigators to be different with different strains of rats. In the Sherman strain McClure found that neither the incidence nor the extent of dental caries was greater when the animals were exposed to the influence of the cariogenic diet in the prenatal state than when feeding of this diet was started at birth. Sognnaes, on the other hand, observed in the Long-Evans strain a definite prenatal influence of diet.

Experiments conducted in our laboratories on the albino rat of the Wistar strain have yielded results that are in some respects similar to and in other respects unlike those obtained by Sognnaes and by McClure.

EXPERIMENTAL

The albino rats of the Wistar strain used in these experiments had been inbred in our laboratories for over 17 years. The experiment consisted of two parts. The first was designed

[1] The expense of this investigation was defrayed in large part by a grant-in-aid from the Sugar Research Foundation.

for making comparative observations on the cariogenicity of different diets; the second to determine whether these diets had a prenatal influence on susceptibility to caries.

Part I

Twenty-four groups of litter mates, of 4 animals each (a total of 96 animals), were selected at weaning from parents that had been raised and maintained on a stock diet. They were then placed on 4 different diets, which will be designated as high sucrose, high protein, high fat, and stock. The composition of the first three of these diets is presented in table 1. The stock diet consisted solely of a commercial animal food.[2] It should be noted that both the high protein and high fat diets contained over 20% sucrose.

At the end of 150 days on the experimental diets the animals were sacrificed and the teeth examined under the microscope for carious lesions. A tabulation of the observations made on these teeth is presented in the upper portion of table 2. In this table we have recorded the number and extent of the carious lesions instead of the usual caries score employed by many investigators. The caries score, obtained by multiplying the average grade of the lesions by the average number of areas affected, is undoubtedly useful for comparative purposes, but we believe a better over-all picture is obtained from more complete data as recorded in table 2.

Caries always started in the fissures and, with very few exceptions, occurred only in the lower molars. The upper molars have therefore been disregarded and the tabulation limited to data on the lowers.

Since there are three fissures in the first lower molar, two in the second and one in the third, each animal presented the possibility of 12 lesions in the lower molars; the 24 animals in each group, 288 possibilities. This should be kept in mind in evaluating the data in table 2.

[2] Purina Laboratory Chow.

A first molar tooth that was completely destroyed by caries was recorded as having three basic lesions; a second molar, two; and a third molar, one lesion. The extent of the lesion in each fissure (see table 2) was graded according to the following standards:

TABLE 1

Composition of the high sucrose, high protein and high fat diets

CONSTITUENTS	DIET		
	1	2	3
	%	%	%
Sucrose	64	22	24
Casein	20	62	25
Fat	8	8	43
Yeast and liver extract	4	4	4
Salt mixture	4	4	4

Salt mixture		*Vitamins*[1]	
Calcium (%)	0.60	Thiamine (p.p.m.)	24
Phosphorus (%)	0.38	Riboflavin (p.p.m.)	24
Magnesium (%)	0.053	Niacin (p.p.m.)	32
Potassium (%)	0.72	Pyridoxine (p.p.m.)	10
Sodium (%)	0.40	Pantothenic acid (p.p.m.)	25
Iron (p.p.m.)	480	Folic acid (p.p.m.)	1
Manganese (p.p.m.)	78	Biotin (p.p.m.)	0.12
Copper (p.p.m.)	10	Inositol (p.p.m.)	130
Iodide (p.p.m.)	1.5	Choline (p.p.m.)	3,000
Fluoride (p.p.m.)	0.1	Carotene (p.p.m.)	3
Zinc (p.p.m.)	4	A concentrate (I.U./gm)	10
Sulfur (p.p.m.)	310	Viosterol (U.S.P. units/gm)	3
Aluminum (p.p.m.)	0.16	α-Tocopherol (p.p.m.)	300
		Linoleic acid (p.p.m.)	500

[1] From yeast and liver extract and supplementary addition. To make the diets more palatable 1.6 p.p.m. butter flavor was added.

Grade 1. Initiation of caries, as indicated by the characteristic brown stain with or without a small cavity.

Grade 2. Cavity large enough to take an "explorer."

Grade 3. Moderate spread of the lesion involving the cusps.

Grade 4. Spread of the lesion from one fissure to another by the breakdown of the cuspal walls.

Grade 5. Spread of the lesion to the extent that the involved area (which might be the entire tooth) was lost.

Part II

Animals were selected from litters that could provide 4 males and 4 females, the parents having been raised and maintained on the stock diet. These young rats were placed at weaning on the same 4 diets as the animals in part I of the experiment. At the age of 100 days brothers were mated with

TABLE 2

Dental caries in the albino rat (Wistar strain) on a high sucrose, a high protein, a high fat and a stock diet

DIET	NO. ANIMALS	ANIMALS WITH CARIES	TOTAL NO. OF CARIOUS MOLARS	TOTAL NO. OF CARIOUS LESIONS	GRADE OF LESION				
					1	2	3	4	5
					Number of lesions				
			First generation						
High sucrose	24	12	22	24	14	8	2	0	0
High protein	24	2	6	7	5	2	0	0	0
High fat	24	4	8	8	8	0	0	0	0
Stock	24	4	6	6	5	1	0	0	0
			Second generation						
High sucrose	24	14	29	29	20	9	0	0	0
High protein	24	1	1	1	1	0	0	0	0
High fat	24	3	4	4	4	0	0	0	0
Stock	24	2	3	3	3	0	0	0	0

sisters on the same diet. The offspring of these animals were designated as second generation animals. When weaned they were fed the same diets as their parents for a period of 150 days. At the end of this period they were sacrificed and the teeth examined under the microscope for caries. The data on these teeth are presented in the second half of table 2.

RESULTS AND DISCUSSION

In several of the animals on the stock diet, as is shown in table 2, a few carious lesions occurred in the lower molars.

The relative frequency, however, was low and the extent of the lesions extremely small.

On the high protein and high fat diets there were a few carious lesions in both the first and second generation animals; but, both as regards incidence and extent, these were of the same order of magnitude as those of animals on the stock diet. It may, therefore, be concluded that the presence of sugar in the diet, amounting to 22% to 24% of the total food mixture, did not make the diets more cariogenic than the ordinary stock diet.

Increasing the amount of sugar to the extent that it comprised 64% of the food mixture resulted in a higher incidence of, and more extensive, dental caries. However, it is apparent from the data in table 2 that the actual number of lesions and the actual destruction of the teeth were small. In this respect our results are somewhat different from those of Sognnaes ('48), using the Long-Evans strain of rats on the Harvard high sucrose diet. In no instance did our animals develop carious lesions that were in any way comparable to the carious destruction which he observed, and reported in photographic illustrations ('49).

Since both diets contained approximately the same amount of sugar, we are confronted with some questions of basic significance. Is the difference in results on the two diets due to hereditary factors in the two strains of rats or is it due to differences in the composition of the diets other than their sugar content? Comparison of the Emory diet (table 1) with the Harvard diet (Sognnaes, '48, p. 677) reveals a number of differences in the mineral and vitamin content.

Should the difference in results be traced to a difference in hereditary factors, there would remain to be determined the nature of these factors. Conceivably there could be a difference in the composition or the apatite structure of the teeth of our Wistar strain animals and those of the Long-Evans strain. Should the difference in the cariogenicity of the two diets, however, be traced to differences in their composition, we would be driven to look for one or more specific

substances in the Emory diet that make the teeth more resistant to caries. These questions are now the subject of joint investigations in the Harvard and the Emory laboratories.

There is still another difference between our results and those of Sognnaes, the cause of which should be investigated. In the experiments of Sognnaes the prenatal diet seemed to be an important predisposing factor with respect to the subsequent dental caries. Considerably greater tooth destruction occurred on a high sucrose diet when the animals were subjected to the prenatal influence of the diet by feeding this diet to the mother before and during pregnancy. Some of the results of Sognnaes' experiments also suggested that the prenatal effects of the diet may have been more pronounced the longer the time the mother was on the diet. Our animals on the high sucrose diet in the second generation were born of parents that had been on this diet from the time of weaning, yet it will be noted from the data in table 2 that there was no greater carious destruction of their teeth than of the teeth of the first generation animals that had not been subjected to the prenatal influence of the diet. In this respect our animals resembled those of the Sherman strain studied by McClure ('51).

It is recognized that more exacting experimental conditions, as suggested by Sognnaes, would be brought about by having two successive litters born of the same parents and placing the mothers on different diets during the two pregnancies. We do not feel, however, that these stringent conditions were called for in this experiment, because of the homozygosity of the animals from which our colony originated and the constant inbreeding over 17 years. In all this time we have never had one animal born in the colony with pigmentation in the eye or colored strands of hair.

McClure found that more of his animals developed dental caries, and that the carious lesions were more severe, when the feeding of the cariogenic diet was begun at birth than when it was begun at weaning. These results are in keeping

with those of Sognnaes. In our experiments, however, using the Wistar strain, there was, as is shown in table 2, no greater carious destruction of the teeth of animals of the second generation that had been fed the high sucrose diet from birth than of those animals that had been fed the same diet from weaning. This would suggest the advisability of further investigation into the possibility of a strain difference in response to the systemic influence of cariogenic diets.

SUMMARY

The number and extent of carious lesions in the teeth of albino rats of the Wistar strain on a high protein and a high fat diet containing 22% to 24% sugar were extremely small and of the same order of magnitude as in animals fed an ordinary stock diet. Increasing the sugar content of the diet so that it constituted 64% of the food mixture resulted in some increase in the carious damage to the teeth. The actual number and the actual extent of the lesions, however, were relatively small. Exposure of the animals to the possible systemic influence of a high sucrose diet in prenatal life did not increase the incidence of dental caries.

LITERATURE CITED

McClure, F. J. 1951 Experimental dental caries in rats. J. Nutrition, *43:* 303.

Sognnaes, R. F. 1948 Caries-conducive effect of a purified diet when fed to rodents during tooth development. J. Am. Dent. Assn., *37:* 676.

———— 1949 Experimental rat caries. II. Location, sequence and extent of carious lesions produced in the Norway rat when raised on a generally adequate, finely powdered, purified ration. J. Nutrition, *39:* 139.